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(54) Title: USE OF TRANSCRIPTION FACTOR BRN-3A

(57) Abstract

The present invention provides a polypeptide comprising transcription factor Brn-3a or a derivative thereof for use in a method of treatment of the human or animal body by therapy.

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USE OF TRANSCRIPTION FACTOR BRN-3A

Field of the invention

This invention relates to the use of transcription factor Brn-3a and derivatives thereof.

Background to the invention

During the normal development of the nervous system, a high proportion of the neurons which initially form undergo programmed cell death or apoptosis. Such cell death is essential for the normal development of the nervous system and is an active process in which the cell effectively commits suicide in an energy dependent process. It is thus distinct from necrotic death induced by toxic or noxious agents which does not normally occur in the developing nervous system.

Although essential for normal development, excessive cell death is also characteristic of a number of human neurological diseases. Thus, several inherited diseases such as familial dysautonomia or infantile spinal muscular atrophy involve enhanced programmed cell death during development resulting in reduced numbers of specific neuronal cell types remaining. Similarly, progressive enhanced neuronal cell death is characteristic of amyotrophic lateral sclerosis (motor neuron disease) and the late onset neurodegenerative diseases such as Alzheimer's and Parkinson's diseases where it is responsible for the progressive neurological lesions which develop.

As well as its role in normal development and in congenital or chronic diseases, programmed cell death is also involved in the response of the nervous system to acute injury. Thus, for example, following spinal injury, the initial death of a number of neurons by necrosis is followed by a subsequent wave of apoptosis that results in the death of further neurons. It is the loss of many neurons in this way coupled with the failure of the remaining neurons to regenerate their axons, which results in the motor and sensory impairment suffered by human spinal injury patients with permanent paralysis below the injured area. Thus, programmed cell death plays a key role in congenital, acute and chronic diseases of the nervous system. An understanding of the mechanisms of this process and in particular, the identification of the means of controlling and manipulating it would therefore be invaluable in developing new methods of treating such diseases.

The three members of the Brn-3 family of transcription factors, Brn-3a, Brn-3b

and Brn-3c. are expressed in distinct but overlapping sets of neurons in the developing and adult nervous systems (Fedtsova *et al.*, 1996). Brn-3a, Brn-3b and Brn-3c are members of the POU (Pit-Oct-Unc) family of transcription factors originally defined on the basis of a common DNA-binding POU domain found in the mammalian transcription factors Pit-1, Oct-1 and Oct-2 and in the nematode factor Unc-86. The original members of this family are known to play a key role in the development of specific neuronal cell types with the Pit-1 factor being essential for the development of the pituitary gland whilst the *unc-86* mutation results in the absence of specific neuronal cell types in the nematode. The observation that all three isoforms of Brn-3 are differentially expressed during mouse neural development suggests roles for these proteins in the regulation of phenotypic changes that occur during neuronal differentiation.

Brn-3a, Brn-3b and Brn-3c show close homology to each other within the DNA-binding POU domain but much less homology outside it, and are encoded by three different genes (Theil *et al.*, 1993 & 1994). Brn-3a is expressed as two different splice variants. The short form, Brn-3aS lacks the N-terminal 84 amino acids of the full-length form, Brn-3aL.

Summary of the invention

The present invention is based on our finding that over expression of transcription factor Brn-3a can protect neuronal cells from apoptosis. We have also shown that Brn-3a activates specifically expression of the Bcl-2 gene in neuronal cells and that this activation is mediated via a Brn-3a response element in the 5' regulatory region of the *Bcl-2* gene. Importantly, our data indicate that both the anti-apoptotic effect of Brn-3a and its ability to activate expression of Bcl-2 appear to be mediated by the N-terminal domain which is present in the full-length Brn-3aL but not in the truncated Brn-3aS.

The members of the Bcl-2 family, which include Bcl-2, Bcl-xL, Bad and Bax, perform critical roles in the regulation of selective apoptosis during development of the nervous system. The stimulation of Bcl-2 expression by Brn-3a in a neuron-specific manner and consequent protection of neuronal cells from apoptosis suggests that Brn-3a may co-ordinate some aspects of neuronal reorganisation during development and following injury. Thus, the elevation of Brn-3a expression by either pharmacological or by gene therapy procedures represents a potential method for treating human diseases involving excessive neuronal death and/or lack of nerve regeneration.

Accordingly, the present invention provides a polypeptide comprising transcription factor Brn-3a or a derivative thereof for use in a method of treatment of the human or animal body by therapy. Preferably, said derivative of Brn-3a comprises an N-terminal fragment, more preferably the N-terminal 84 amino acids of the full-length form of Brn-3a. More preferably, said derivative possesses anti-apoptotic activity but not neurite outgrowth stimulating activity.

The present invention further provides a polynucleotide encoding said Brn-3a or derivative thereof for use in a method of treatment of the human or animal body by therapy. The present invention also provides a nucleic acid vector comprising a polynucleotide encoding said Brn-3a or derivative thereof operably linked to regulatory sequences permitting expression of Brn-3a or a derivative thereof in a host cell. Preferably the host cell is a cell of the central or peripheral nervous system of an animal, more preferably a mammal, including primates and humans.

The nucleic acid vectors of the present invention may also comprise mammalian genomic sequences flanking said polynucleotide and regulatory sequences to allow introduction of the polynucleotide into the genome of a mammalian cell by homologous recombination. Preferably said mammalian genomic sequences are derived from the human genome. The vectors may also comprise viral genomic sequences flanking said polynucleotide and regulatory sequences to allow construction of viral strains which comprise a polynucleotide encoding said Brn-3a or derivative thereof. Preferably said viral genomic sequences are derived from the genome of a herpes simplex virus.

Thus the present invention further provides a viral strain comprising a polynucleotide encoding Brn-3a or a derivative thereof operably linked to regulatory sequences permitting expression of said Brn-3a or derivative thereof in a host cell, for use in a method of treatment of the human or animal body by therapy. Preferably said viral strain is a herpes simplex virus.

Delivery of the polynucleotides, vectors or viral strains of the invention to a host cell may be enhanced by combining them with a transfection agent. Such transfection agents include cationic agents (for example calcium phosphate and DEAE-dextran) and liposome compositions (for example Lipofectin™ and Transfectam™). Thus the present invention also provides a composition comprising a polynucleotide, a vector or a viral strain of the invention together with a transfection agent, for use in a method of treatment of the human or animal body by therapy.

Treatment of neurodegenerative diseases and nerve injuries may require administration of additional therapeutic polypeptides. For example, neurotrophic factors including nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BNTF) and neurotrophins (such as NT-3, NT-4/5) have potential as therapeutic agents for the treatment of Parkinson's disease. Thus the present invention provides a composition comprising a polypeptide according to the invention and at least one other therapeutic polypeptide, for use in a method of treatment of the human or animal body by therapy.

The therapeutic polypeptide may also be administered in the form of a polynucleotide encoding said therapeutic polypeptide and said polynucleotide may form part of a nucleic acid vector or viral strain in the same manner as for the polynucleotide encoding Brn-3a or a derivative thereof.

Diseases which may be treated using the polypeptides, polynucleotides, vectors, viral strains and compositions of the invention include diseases of the peripheral or central nervous system such as neurodegenerative diseases and damage to nervous tissue as a result of injury/trauma. In particular, neurodegenerative diseases include motor neurone disease, several inherited diseases, such as familial dysautonomia and infantile spinal muscular atrophy, and late onset neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.

The polypeptides, polynucleotides, vectors, viral strains and compositions of the invention may in particular be used in preventing apoptosis in a cell of the peripheral or central nervous system of a human or animal and/or in enhancing nerve regeneration following damage, typically due to injury.

Brief description of the figures

Figure 1 shows graphs comparing cell survival/apoptosis in the presence or absence of Brn-3a over expression.

Figure 2a illustrates the constructs used in this study.

Figure 2 b depicts graphs of cell survival for cells expressing different Brn-3a constructs.

Figure 3a is a Western blot of ND7 cell extracts showing the levels of Bcl-2 and Bad in response to Brn-3a expression.

Figure 3b is a graph depicting Bcl-2 expression in response to over expression of Brn-3a.

Figure 4a illustrates the 5' end of the Bcl-2 gene.

Figures 4b,c show the results of reporter gene assays using different Brn-3a constructs.

Figure 5 shows the results of reporter gene assays using different Bcl-2 promoter constructs.

Figure 6 shows the results of reporter gene assays using different Brn-3 factors and a region of the Bcl-2 promoter.

Figure 7 depicts graphs of cell survival in the presence or absence of Brn-3a.

Figure 8 shows the survival of trigeminal ganglion neurons obtained from various stages of embryonic development, in the presence or absence of Brn-3a over expression.

Figure 9 shows the survival of trigeminal ganglion neurons obtained from various stages of embryonic development, in the presence or absence of an anti-sense Brn-3a vector.

Detailed description of the invention

A. Polypeptides

Polypeptides of the invention comprise transcription factor Brn-3a and derivatives thereof. Both murine and human forms of Brn-3a have been cloned and their nucleotide and amino acid sequences determined. The human Brn-3a sequence is published in Xiang *et al.*, 1995 (Genbank accession numbers U10062/U10063/ U09783) and is shown in SEQ. ID. Nos. 1 and 2. The sequence of murine Brn-3a is published in Theil *et al.*, 1994 (Genbank accession number S69350) and is shown in SED. ID. Nos. 3 and 4 (The introns have been removed from SEQ. ID. No. 3).

Thus a polypeptide of the invention comprises the amino acid sequence set out in SEQ ID Nos. 2 or 4 or a substantially homologous sequence, or of a fragment of either of these sequences. In general, the naturally occurring human or murine Brn-3a amino acid sequence shown in SEQ ID Nos. 2 and 4, respectively, are preferred. However, the polypeptides of the invention include derivatives of the natural human or murine Brn-3a sequences. The term derivative is taken to encompass all homologues and allelic variants having substantial homology to the human or murine Brn-3a sequences set out in SEQ ID. Nos. 2 and 4. It is also taken to include fragments which possess anti-apoptotic activity of the human or murine Brn-3a sequences set out in SEQ ID. Nos. 2 and 4, and of their homologues and allelic variants. In particular, preferred derivatives will comprise an amino acid sequence having substantial homology to the N-terminal 84 amino acids

present in the full-length splice variant of mouse Brn-3a (amino acids 1 to 84 of SEQ. ID. No. 4 encoded by nucleotides 1 to 234 of SEQ. ID. No. 3) or human Brn-3a (amino acids 1 to 84 of SEQ. ID. No. 2 encoded by nucleotides 1 to 234 of SEQ. ID. No 1). It should be noted that the N-terminal 98 amino acids of mouse and human Brn-3a are identical, with only one difference in the next 37 residues up to amino acid 135. Thus the N-terminal region of Brn-3a is highly conserved between mice and humans.

An allelic variant will be a variant which will occur naturally in a human or mouse and which will function in a substantially similar manner to the polypeptide of SEQ ID Nos. 2 or 4. In particular, it will have anti-apoptotic activity. Similarly, a species homologue of the protein will be the functionally equivalent polypeptide which occurs naturally in another species. Such a homologue may occur in animals such as mammals (e.g. rats or rabbits), especially primates. Within any one species, a homologue may exist as several allelic variants, and these will all be considered homologues of the protein of SEQ ID Nos. 2 and 4.

Allelic variants and species homologues can be obtained using standard procedures. In particular, it will be possible to use a Brn-3a nucleotide sequence to probe libraries made from mammalian cells to obtain clones encoding the allelic or species variants. The clones can be manipulated by conventional techniques to identify a polypeptide of the invention which can then be produced by recombinant or synthetic techniques known *per se*. Preferred species homologues include primate species homologues.

A protein at least 70% homologous to the polypeptide of SEQ ID Nos. 2 or 4 or an allelic variant or species homologue thereof will be preferably at least 80 or 90% and more preferably at least 95%, 97% or 99% homologous thereto over a region of at least 20, preferably at least 30, for instance at least 40, 60 or 100 or more contiguous amino acids. In particular, an allelic variant or species homologue will be at least 90, 95%, 97% or 99% homologous over the N-terminal domain of at least 40, 60 or 100 contiguous amino acids. Methods of measuring protein homology are well known in the art and it will be understood by those of skill in the art that in the present context, homology is calculated on the basis of amino acid identity (sometimes referred to as "hard homology").

Further, the sequence of the polypeptide of SEQ ID Nos. 2 and 4 and of allelic variants and species homologues can be modified to provide polypeptides of the

invention. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions provided that the modified polypeptide retains anti-apoptotic activity.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

Derivatives of the polypeptides of the invention will preferably also possess neurite outgrowth stimulating activity. We have shown that this activity is primarily located in the C-terminal DNA-binding POU domain. Thus the polypeptides of the invention preferably comprise a domain having substantial homology to the C-terminal DNA-binding POU domain of human or murine Brn-3a , more preferably comprising approximately amino acids 302 to 416 as set out in SEQ ID. Nos 2 or 4.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

We have also shown that specific mutations in the N-terminal domain and C-terminal domain of Brn-3a can abolish the anti-apoptotic and neurite outgrowth stimulating activity of Brn-3a, respectively. Thus it is possible to prepare polypeptides which lack one of these activities by mutagenesis techniques including substitutions, insertions and deletions. In particular, an amino acid substitution of valine by isoleucine at position 22 of the homeodomain of murine Brn-3a (residue 380 of SEQ. ID. No. 4, equivalent to residue 378 of SEQ. ID. No. 2) significantly reduces the neurite outgrowth stimulating activity of Brn-3a. This may also be achieved by replacing the C-terminal domain of Brn-3a with the equivalent C-terminal domain of Brn-3b (which has an isoleucine residue at position 22 of the homeodomain in place of a valine residue). Further, deletion of the N-terminal domain of Brn-3a present in the full-length splice

variant but not the short variant abolishes the ability of Brn-3a to inhibit apoptosis. Alternatively, derivatives of Brn-3a which possess anti-apoptotic activity but not neurite outgrowth stimulating activity may be prepared by fusing the N-terminal domain of Brn-3a to a heterologous DNA binding domain, typically a POU domain, capable of binding to the promoter region of the *bcl-2* gene. The preparation and testing of such a fusion protein can be performed using routine techniques of molecular biology.

Thus the polypeptides of the invention may be used to prepare a variety of therapeutic reagents which can either stimulate neurite outgrowth or inhibit apoptosis, or both. Preferably, the polypeptides of the invention exert specifically their effects in neuronal cells, more preferably in sensory or motor neuronal cells.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the polypeptide in the preparation is a polypeptide of the invention.

Polypeptides of the invention may be made by synthetic means or recombinantly using techniques well known to skilled persons.

Polypeptides of the invention may be used in *in vitro* or *in vivo* cell culture systems to study the role of Brn-3a, and its homologues in disease. For example, truncated or modified Brn-3a may be introduced into a cell to disrupt the normal functions which occur in the cell. The polypeptides of the invention may be introduced into the cell by *in situ* expression of the polypeptide from a recombinant expression vector (see below). The expression vector optionally carries an inducible promoter to control the expression of the polypeptide.

The use of mammalian host cells is expected to provide for such post-translational modifications (e.g. myristolation, glycosylation, truncation, lapidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention. Such cell culture systems in which polypeptide of the invention are expressed may be used in assay systems to identify candidate substances which interfere with or enhance the functions of the polypeptides of the invention in the cell.

B. Assaying anti-apoptotic and neurite outgrowth stimulating activity

The derivatives discussed above can be tested for anti-apoptotic and neurite outgrowth stimulating activity using the following assay methods.

Anti-apoptotic activity

Typically, ND7 cells can be used as a model system for determining if derivatives of Brn-3a inhibit apoptosis. Apoptosis can be induced in ND7 cells by transferring the cells to serum free media, optionally containing retinoic acid (RA).

The induction of apoptosis can be determined by various means. There are several techniques known to a skilled person for determining if cell death is due to apoptosis. Apoptotic cell death is characterised by morphological changes which can be observed by microscopy, for example cytoplasmic blebbing, cell shrinkage, internucleosomal fragmentation and chromatin condensation. DNA cleavage typical of the apoptotic process can be demonstrated using TUNEL assays and DNA ladder assays (see Materials and Methods, Gavreili *et al.*, 1992).

Typically, the derivative of Brn-3a is stably integrated in the genome of ND7 cells. This can be achieved by transfecting cells with a nucleic acid encoding the Brn-3a under the control of a suitable promoter (for example the mouse mammary tumour virus (MMTV) promoter) as well as a selective marker, for example the neomycin resistance gene. A certain population will take up the nucleic acid and a subpopulation of these will integrate the nucleic acid into their genome. These stable clones can subsequently be selected (in the case of the marker being the neomycin resistance gene, by the addition on geneticin (G418) to the culture medium).

Preferably, the apoptotic response to serum starvation/RA is reduced in the presence of the derivative by at least 25% relative to the apoptotic response in the absence of derivative, more preferably by at least 50% or 75%. So, for example, if the percentage cell survival of ND7 cells in the absence of the derivative is 20%, then the percentage cell survival in the presence of the derivative is preferably greater than 40%, more preferably greater than 60% or 80%.

Neurite outgrowth stimulating activity

Typically, ND7 cells can be used as a model system for determining if derivatives of Brn-3a stimulate neurite outgrowth. Neurite outgrowth length can be determined using

immunocytochemical techniques (as described in Smith *et al.*, 1997 a.b). Cells are incubated with a primary antibody to α-tubulin with detection using the peroxidase-diaminobenzidene colour reaction. The length of the longest process for each of 400 cells is then determined for each sample using video capture NIH Image software (version 1.59).

A construct expressing the derivative, under the control of an inducible promoter (for example the MMTV promoter inducible with dexamethasone), is stably integrated into the genome of the ND7 cells as described above. A control vector lacking sequences encoding the derivative is used as a control. The number and lengths of processes formed in the absence or presence of the inducer (for example 1 μM dexamethasone) is then measured for cells transfected with the control vector versus cells transfected with the derivative-expressing vector.

Preferably, the percentage number of cells with neurites in the presence of the derivative is increased by at least 50% relative to the percentage number of cells with neurites with the control vector alone, more preferably by at least 75% or 100%. Preferably, the average neurite length of cells in the presence of the derivative is increased by at least 50% relative to the average neurite length of cells with the control vector alone, more preferably by at least 75% or 100%.

C. Polynucleotides and vectors.

Polynucleotides of the invention comprise nucleic acid sequences encoding the polypeptides of the invention. Polynucleotides of the invention may comprise DNA or RNA. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention. In a preferred embodiment of the invention, the polynucleotide comprises the nucleic acid sequence as shown in SEQ ID No. 1 or SEQ ID. No. 3.

Preferred polynucleotides of the invention also include polynucleotide encoding any of the polypeptides of the invention described above. It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result

of the degeneracy of the genetic code.

Polynucleotides of the invention comprise can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells.

Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

Such vectors may be transformed or transfected into a suitable host cell as described above to provide for expression of a polypeptide of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and optionally recovering the expressed polypeptides.

The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell. The vector may also be adapted to be used *in vivo*, for example in a method of gene therapy. In particular viral vectors may be used as described below.

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, mammalian promoters, such as β-actin promoters, may be used. Tissues-specific promoters, in particular neuronal cell specific promoters (for example the tyrosine hydroxylase (TH),

L7, or neuron specific enolase (NSE) promoters), are especially preferred. Promoters which are specific for particular neuronal cell types, for example sensory or motor neurons are especially preferred. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the promoter rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters. All these promoters are readily available in the art.

D. Viral vectors

The polynucleotides of the invention may be used in the form of a naked nucleic acid construct. Alternatively, they may be introduced into a variety of nucleic acid vectors. Such vectors include plasmids and viral vectors, preferably herpes simplex virus vectors. Vectors may further include sequences flanking a polynucleotide of the invention which comprise sequences homologous to eukaryotic genomic sequences, preferably mammalian genomic sequences, or viral genomic sequences. This will allow the introduction of the polynucleotides of the invention into the genome of eukaryotic cells or viruses by homologous recombination. In particular, a plasmid vector comprising the expression cassette flanked by viral sequences, preferably HSV1 or HSV2 sequences, can be used to prepare a viral vector, preferably an HSV vector, suitable for delivering the polynucleotides of the invention to a mammalian cell. This is described in further detail below for the herpes simplex virus. However the techniques employed are well-known to a skilled person and will be suitable for other viruses such as adenoviruses. Other examples of suitable viral vectors include viral vectors able to integrate their genomes into the host cell genome, for example retroviruses, including lentiviruses, and adeno-associated virus.

Herpes Simplex Virus Vectors

1. Viral Strains

The viral strains of the invention comprising a polynucleotide encoding Brn-3a or a derivative thereof may be derived from, for example, HSV1 or HSV2 strains or derivatives thereof, preferably HSV1. Derivatives include inter-type recombinants containing DNA from HSV1 and HSV2 strains. Derivatives preferably have at least 80%

sequence homology to either the HSV1 or HSV2 genomes, more preferably at least 90%, even more preferably 95%.

The use of HSV strains in therapeutic procedures will require the strains to be attenuated so that they cannot establish a lytic cycle. In particular, if the HSV vectors are to be used for gene therapy in humans the polynucleotide of the invention should preferably be inserted into an essential gene. This is because if a vector virus encounters a wild-type virus transfer of the polynucleotide of the invention to the wild-type virus could occur by recombination. However as long as the polynucleotide of the invention is inserted into an essential gene this recombinational transfer would also delete the essential gene in the recipient virus and prevent 'escape' of the heterologous gene into the replication competent wild-type virus population.

Attenuated strains which may be used to produce HSV strains suitable for use in gene therapy, given as examples only, include strains that have mutations in ICP34.5 or ICP27, for example strain 1716 (MacLean *et al.*, 1991), strains R3616 and R4009 (Chou and Roizman, 1992) and R930 (Chou *et al.*, 1994) all of which have mutations in ICP34.5, and d27-1 (Rice and Knipe, 1990) which has a deletion in ICP27. Strains deleted for both ICP34.5 and ICP27 may also be used, as disclosed in UK Application No. 9615794.6. Alternatively, ICP4, ICP0, ICP22, VHS or gH, with an inactivating mutation in VMW65, or with any combination of the above may also be used to produce the HSV strain of the invention.

The terminology used in describing the various HSV genes is as found in Coffin and Latchman, 1996.

2. Complementing cell lines

HSV viruses defective in ICP27 are propagated in a cell line expressing ICP27, for example V27 cells (Rice and Knipe, 1990), 2-2 cells (Smith *et al.*, 1992) or B130/2 cells, preferably B130/2 cells (see below).

ICP27-expressing cell lines can be produced by co-transfecting mammalian cells, for example the Vero or BHK cells, with a vector, preferably a plasmid vector, comprising a functional HSV ICP27 gene capable of being expressed in said cells, and a vector, preferably a plasmid vector, encoding a selectable marker, for example neomycin resistance. Clones possessing the selectable marker are then screened further to determine which clones also express functional ICP27, for example on the basis of their

ability to support the growth of ICP27⁻ mutant HSV strains, using methods known to those skilled in the art (for example as described in Rice and Knipe, 1990).

A suitable complementing cell line (B130/2) allowing growth of ICP27 deleted viruses and having no overlap between the complementing sequences and the ICP27 deleted viruses above (and thus preventing repair of ICP27 by homologous recombination during virus growth) is described in UK patent application no. 9615794.6. B130/2 was generated by co-transfection of plasmid pSG130BS (Sekulovich *et al.*, 1988) DNA with neomycin resistance-encoding plasmid pMamNeo (Invitrogen) into BHK cells and the selection of neomycin resistant clones. A clone (B130/2) highly permissive for the growth of an HSV-1 ICP27 deletion mutant was selected for virus growth. PSG130BS carries a BamHI/SacI fragment from HSV-1 (nts 113322-115743) encoding the complete ICP27 coding sequence and part of UL55.

Cell lines which do not allow reversion of an ICP27⁻ mutant HSV strain to a strain with functional ICP27 are produced as described above, ensuring that the vector comprising a functional ICP27 gene does not contain sequences that overlap with (i.e. are homologous to) sequences remaining in the ICP27⁻ mutant virus.

Where HSV strains comprise inactivating modifications in other essential genes, for example ICP4, complementing cell lines will further comprise a functional HSV gene which complements the modified essential gene in the same manner as described for ICP27.

3. Methods of mutation

HSV genes may be rendered functionally inactive by several techniques well known in the art. For example, they may be rendered functionally inactive by deletions, substitutions or insertions, preferably by deletion. Deletions may remove portions of the genes or the entire gene. Inserted sequences may include the expression cassette described above.

Mutations are made in the HSV strains by homologous recombination methods well-known to those skilled in the art. For example, HSV genomic DNA is transfected together with a vector, preferably a plasmid vector, comprising the mutated sequence flanked by homologous HSV sequences. The mutated sequence may comprise deletions, insertions or substitutions, all of which may be constructed by routine techniques. Insertions may include selectable marker genes, for example *lacZ*, for screening

recombinant viruses by, for example, β-galactosidase activity.

Mutations may also be made in other HSV genes, for example IE genes such as ICP0, ICP4, ICP6, ICP22, ICP47or VMW65, preferably ICP4 and VMW65. In the case of the VMW65 gene, the entire gene is not deleted since it encodes an essential structural protein, but a small inactivating insertion is made which abolishes the ability of VMW65 to transcriptionally activate IE genes (Ace *et al.*, 1989).

4. HSV strains comprising the expression cassette

A polynucleotide of the invention may be inserted into the HSV genome at any location provided that the virus can still be propagated, which may require the use of a cell line carrying another HSV essential gene (as described in 2.) if the polynucleotide is inserted into an essential gene. For example, if the polynucleotide is inserted into the ICP27 gene of the HSV strain, then a cell-line expressing ICP27 would be needed. The polynucleotide is preferably inserted into the region of the ICP27 mutation as in the unlikely event that the mutation is repaired by recombination with a wild-type virus, the repair would remove the inserted polynucleotide.

The polynucleotide of the invention may be inserted into the HSV genome by homologous recombination of HSV strains with, for example, plasmid vectors carrying the polynucleotide flanked by HSV sequences, as described above for introducing mutations. The polynucleotide may be introduced into a suitable plasmid vector comprising HSV sequences using cloning techniques well-known in the art.

E. Therapeutic polypeptides

Treatment of diseases using the polypeptides, polynucleotide and vectors of the invention may be improved by administering additional therapeutic polypeptides. For example, neurotrophic factors including nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BNTF) and neurotrophins (such as NT-3, NT-4/5) have potential as therapeutic agents for the treatment of Parkinson's diseaseThe therapeutic polypeptide may also be administered in the form of a polynucleotide encoding said therapeutic polypeptide and said polynucleotide may form part of a nucleic acid vector or viral strain in the same manner as for the polynucleotide encoding Bm-3a or a derivative thereof.

F. Administration

The polypeptides, polynucleotides and vectors of the invention, optionally with an additional therapeutic polypeptide or nucleic acid/vector encoding said therapeutic polypeptide, may thus be administered to a human or animal in need of treatment. Diseases which may be treated using the polypeptides, polynucleotides, vectors, viral strains and compositions of the invention include diseases of the peripheral or central nervous system such as neurodegenerative diseases and damage to nervous tissue as a result of injury/trauma (including strokes). In particular, neurodegenerative diseases include motor neurone disease, several inherited diseases, such as familial dysautonomia and infantile spinal muscular atrophy, and late onset neurodegenerative diseases such as Parkinson's and Alzheimer's diseases.

The polypeptides of the invention and compositions comprising polypeptides of the invention together with at least one therapeutic polypeptide may be administered by direct injection into the site to be treated, for example tissue of the nervous system. Preferably the polypeptides are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration. Typically, each polypeptide is administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg.

The polynucleotides of the invention may be administered directly as a naked nucleic acid construct, preferably further comprising flanking sequences homologous to the host cell genome. Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™). Typically, nucleic acid constructs are mixed with the transfection agent to produce a composition.

Preferably the naked nucleic acid construct, viral vector comprising the polynucleotide or composition is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or

transdermal administration.

The pharmaceutical composition is administered in such a way that the polynucleotide of the invention, viral vector for gene therapy, can be incorporated into cells at an appropriate area. For example, when the target of gene therapy is the central or peripheral nervous system and the polynucleotide of the invention is to be delivered by a herpes simplex virus vector, the composition could be administered in an area where synaptic terminals are located so that the virus can be taken up into the terminals and transported in a retrograde manner up the axon into the axonal cell bodies via retrograde axonal transport. The pharmaceutical composition is typically administered to the brain by stereotaxic inoculation. When the pharmaceutical composition is administered to the eye, sub-retinal injection is typically the technique used.

When the polynucleotide of the invention is delivered to cells by a viral vector, the amount of virus administered is in the range of from 10^4 to 10^8 pfu, preferably from 10^5 to 10^7 pfu, more preferably about 10^6 pfu for herpes viral vectors and from 10^6 to 10^{10} pfu, preferably from 10^7 to 10^9 pfu, more preferably about 10^8 pfu for adenoviral vectors. When injected, typically 1-2 µl of virus in a pharmaceutically acceptable suitable carrier or diluent is administered. When the polynucleotide of the invention is administered as a naked nucleic acid, the amount of nucleic acid administered is typically in the range of from 1 µg to 10 mg.

Where the polypeptide of the invention is under the control of an inducible promoter, it may only be necessary to induce gene expression for the duration of the treatment. Once the condition has been treated, the inducer is removed and expression of the polypeptide of the invention ceases. This will clearly have clinical advantages. Such a system may, for example, involve administering the antibiotic tetracycline, to activate gene expression via its effect on the tet repressor/VP16 fusion protein.

The use of tissue-specific promoters will be of assistance in the treatment of disease using the polypeptides, polynucleotide and vectors of the invention. For example, several neurological disorders are due to aberrant expression of particular gene products in only a small subset of cells. It will be advantageous to be able express therapeutic genes in only the relevant affected cell types, especially where such genes are toxic when expressed in other cell types. Examples of neuronal sub-types which may be targeted specifically include neurons of the peripheral nervous system, for example sensory neurones, motor neurons, autonomic neurons such as sympathetic neurons and

parasympathetic neurons, and neurons of the central nervous system, for example motor neurons or sensory neurons.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

The invention will be described with reference to the following Examples which are intended to be illustrative only and not limiting. The Examples refer to the Figures. Referring to the Figures in more details:

Figure 1 Brn-3a expression rescues ND7 cells from apoptotic programmed cell death following serum removal.

a) Cell survival in cell lines over expressing Brn-3a (filled squares) or Brn-3b (open circles) maintained in serum free media containing 1 μM RA for 72 hrs, compared to the values in ND7 cells transfected with pJ5 vector alone (open squares).

b) Relative amounts of DNA fragmentation within cells expressing either pJ5 vector alone (open squares), Brn-3a (filled squares) or Brn-3b (open circles) maintained in serum free media containing 1 μM RA for 24, 48 and 72 hrs.

c) TUNEL positive nuclei as a proportion of the total number ND7 cells expressing either empty pJ5 vector alone or Brn-3a in the absence (stippled bars) or presence (filled bars) of dexamethasone, when maintained in serum free media containing 1μM RA for 48 hrs.

Figure 2 Protection of ND7 cells requires the presence of an N-terminal activation domain within Brn-3a.

a) Schematic representation of the constructs used in this study. Constructs were subcloned into pJ5 expression vector for the generation of stable cell lines, or the pJ4 (Morgenstern and Land, 1990) expression vector for transient expression studies. The chimeric constructs (C1, C2, C3 and C4) were not subcloned into pJ5.

b) Cell survival in cell lines over expressing long form Brn-3a (filled squares), the short form of Brn-3a (open circles), or the isolated POU domain of Brn-3a (filled circles) when maintained in serum free media containing 1 μM RA for 24, 48 and 72 hrs and following induction of the MMTV promoter with dexamethasone compared to the value in

ND7 cells transfected with pJ5 vector (open squares).

Figure 3 Bcl-2 protein expression is regulated by Brn-3a in ND7 cells.

a) Western blot analysis of Bcl-2 (top panel) and Bad (middle panel) protein expression in extracts from cell lines over expressing the indicated construct when maintained in full serum media in the absence (-) or presence (+) of dexamethasone. Equal loading of samples was confirmed by detection of pGp9.5 protein levels (lower panel).

b) Bcl-2 protein expression in cell lines expressing the indicated constructs compared to that in ND7 cells transfected with vector alone (pJ5) maintained in the absence (stippled boxes) or presence (filled boxes) of dexamethasone.

Figure 4 The human Bcl-2 promoter is regulated by the N-terminal activation domain of the Brn-3a transcription factor.

a) Schematic representation of the 5' end of the human Bcl-2 gene indicating the two promoter regions (P1 and P2). ORF indicates the start of the open reading frame; hatched regions indicate introns; x indicates potential Brn-3 binding sites.

b, c) Results of the reporter gene assay following the co-transfection into ND7 cells (filled boxes) or BHK cells (stippled boxes) of the indicated plasmids with (b) P1, (c) P2 Bcl-2 promoter constructs. C1, C2, C3 and C4 indicate the chimeric constructs of Brn-3a and Brn-3b drawn schematically in Fig 2a.

Figure 5 Deletion constructs of P1 and P2 Bcl-2 promoters identify regions necessary for activation of reporter constructs by Brn-3a.

a) Results of luciferase assays following the co-transfection of the long form of Brn-3a with the indicated deletion constructs of the human P1 Bcl-2 promoter identify a 113bp fragment (-1528 to -1416) necessary for activation.

b) Results of luciferase assays following the co-transfection of the long form of Brn-3a with the indicated deletion constructs of the human P2 Bcl-2 promoter regions identify a 569 bp region (-746 to -178) required for activation. Note the 8-fold increase in activation of the P2 region following deletion of the sequence between -3933 and -1793 which includes the P1 region. Stippled boxes are the appropriate vector control transfections for each deletion construct.

Figure 6 Identification of a motif within the human Bcl-2 promoter region that is bound by Brn-3a and is sufficient for activation of a heterologous promoter by Brn-3a.

a) Chloramphenical acetyltransferase (CAT) assay of the ability of the indicated Brn-3 factors to transactivate a heterologous promoter containing a 173 bp *BglII/NsiI* fragment from the human P2 Bcl-2 promoter region (-744 to -571).

b) Results of a CAT assay to determine the ability of the indicated Brn-3 factors to transctivate a heterologous promoter containing the minimal CATCAATCTTC motif contained within the *BglII/NsiI* fragment and the OligoP2 (-594 to -584).

Figure 7 Brn-3a over expression protects (a) neonatal dorsal root ganglions and (b) neonatal trigeminal neurons from apoptosis induced by withdrawal of nerve growth factor.

The bars indicate the number of surviving successfully transfected neurons (as determined by staining for the presence of the co-transfected b-galactosidase expressing plasmid). Stippled bars indicate the results in cultures maintained in the presence of NGF and the solid bars in cultures from which NGF was withdrawn. Cells were either injected with parental expression vector or the Brn-3a expression vector as shown.

Figure 8 Survival of trigeminal ganglion neurons obtained from the indicated stage of embryonic development, transfected with either empty expression vector (solid line) or Brn-3a expression vector (dotted line) and then cultured in the presence (panel a) or absence (panel b) of NGF. Values are the mean of at least three independent experiments whose standard deviation is shown by the bars.

Figure 9 Survival of trigeminal ganglion neurons obtained from the indicated stage of embryonic development, transfected with empty expression vector (solid line) or the anti-sense Brn-3a vector (dotted line) and cultured in the presence of NGF. Values are the mean of at least three independent experiments whose standard deviation is shown by the bars.

EXAMPLES

Materials and Methods

Unless indicated otherwise, the methods used are standard biochemical techniques. Examples of suitable general methodology textbooks include Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

DNA ladder assay

Cells were grown on 5 cm tissue culture dishes (Nunc, Roskilde, Denmark). After treatment, the cells were harvested, washed once with ice-cold PBS, resuspended in 250 ml of lysis buffer (10 mM Tris-HCl pH 7.6, 20 mM EDTA pH 8.0, 0.5% v/v Triton X-100) and the samples were left at room temperature for 15 min. After centrifugation at 16,000 g for 5 min, the supernatant was transferred to another eppendorf tube and sequential extractions were carried out. The samples were extracted once with equal volume of phenol, once with phenol-chloroform (1:1) and once with chloroform-isoamylalcohol (24:1). DNA was precipitated with 0. 1 volumes of 3 M sodium acetate pH 4.8 and 2.5 volumes of ethanol at -20°C overnight. The DNA was then pelleted at 16,000 g for 30 min the next day. After washing once with 70% ethanol, the samples were digested with 0.1 μg of RNAase A at 37°C for 30 min before loading onto 1.5% agarose gels and the DNA was visualized by ethidium bromide staining.

TUNEL assay

Measurement of DNA fragmentation by the terminal-deoxynucleotidyl-transferase (TnT) - mediated dUTP-biotin nick end labelling assay (TUNEL) was carried out using the Apop Tag detection kit (Oncor, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, residues of digoxigenin-nucleotides are added to the 3'-OH end of DNA fragments by terminal deoxynucleotidyl transferase and the anti-digoxigenin antibody peroxidase conjugate is used to stain the apoptotic DNA fragments.

Example 1 - Brn-3a rescues ND7 cells from apoptosis

We have used the ND7 neuronal cell line (Wood *et al.*, 1990) as a model system to identify genes whose protein products could protect neuronal cells from apoptosis. These cells were originally isolated by the immortalisation of rat dorsal root ganglion (DRG) neurons and can be grown in large amounts in culture. We have previously reported the construction and characterisation of cell lines derived from the ND7 sensory neuronal cell line that over express high levels of the various members of the Brn-3 family of transcription factors under the control of the glucocorticoid-inducible MMTV promoter (Smith *et al.*, 1997a&b). Upon serum removal parental ND7 cells undergo either apoptotic programmed cell death (PCD) or differentiation to a non-dividing phenotype bearing neuritic processes, whilst retinoic acid (RA) enhances the degree of apoptosis during the differentiation event (Howard *et al.*, 1993).

Following the transfer of the stable cell lines containing the pJ5 vector (Morgenstern and Land, 1990) with no insert to serum free media containing 1 μM dexamethasone to induce the MMTV promoter, a proportion of cells ceased to proliferate and began to extend processes, in agreement with previous studies. The degree of cell death in these control cells was assayed by trypan blue exclusion and found to be similar to that in parental ND7 cells at all timepoints. The addition of 1 μM all-trans RA to the differentiated cultures resulted in a further decrease in the number of viable cells at all timepoints. These data demonstrate that the addition of dexamethasone has no significant effect on the proportion of viable cells when compared to the effects observed in parental ND7 cells under the same experimental conditions. These similarities were observed in all vector-alone control cell lines that were studied. However, significant increases (24hr $p<0.05$; 48hr $p<0.005$; 72hr $p<0.05$) in the number of viable cells, were observed in the three independent cell lines stably overexpressing high levels of full-length Brn-3a (Brn-3aL) following the induction of the MMTV promoter upon the addition of 1 μM dexamethasone to the RA-containing serum-free media (Figure 1a; Table I, Brn-3aL). Similar protection was afforded by over expression of Brn-3a in serum free media that was not supplemented with RA .

TABLE I. Over expression of Brn-3a, but not Brn-3b or Brn-3c, rescues ND7 cell following serum removal.

	Cell survival (Number of viable cells as % of original cell population) [1]					
	24 hours		48 hours		72 hours	
	Dex -	Dex +	Dex -	Dex +	Dex -	Dex +
pJ5	60±6	63±5	49±5	41±8	41±7	44±9
Brn-3aL	63±10	78±6*	61±8	80±9**	54±11	66±8*
Brn-3b	71±12	65±6	40±9	43±5	43±12	38±6
Brn-3aS	55±11	51±7	51±9	45±12	36±6	40±8
Brn-3aP	61±4	55±9	45±6	40±6	47±11	42±8
Brn-3aI	71±5	70±8	57±9	82±5**	52±7	69±4*
Brn-3bV	67±4	65±6	41±6	44±7	42±10	38±6

[1] Cell survival 24, 48 and 72 hours following transfer to serum free media containing 1 μM retinoic acid determined by trypan blue exclusion. Values are means ± standard deviation of the mean determined in duplicate for three independent cell lines expressing each construct. L= long form, S=short form, P=isolated POU domain, I=Isoleucine mutant, V=valine mutant.

* Statistically significant increase from appropriate vector control ($p<0.05$)

** Statistically significant increase from appropriate vector control ($p<0.005$)

Thus as transfer of ND7 cells to serum free media containing RA is known to induce apoptotic PCD, these data show that elevated levels of Brn-3a are capable of rescuing ND7 cells from a fate of apoptotic PCD. Such a reduction in the number of apoptotic cells was confirmed by flow cytometric analysis of PI/FITC stained cells which identified an apoptotic population with less than 2N DNA content in differentiated control ND7 cells (empty vector alone) which was reduced following the induction of Brn-3a expression in the three Brn-3a over expressing lines. No significant alteration in the number of viable cells or in the degree of apoptosis was observed in all cell lines over

expressing Brn-3b (Figure 1a Table 1).

To confirm and extend these observations, we utilised a number of techniques to assay the degree of nucleosomal fragmentation of genomic DNA. The over expression of Brn-3a resulted in a decrease in the proportion of fragmented DNA within the cell populations as determined by both the colormetric DNA fragmentation assay of Sellins and Cohen (1987), as well as the degree of nucleosomal laddering following agarose gel electrophoresis of genomic DNA. No significant alteration in the degree of chromatin fragmentation was observed in cell lines over expressing Brn-3a.

These observations were extended by the labelling of the cultured cells by the TUNEL technique which utilises the labelling of free 3' DNA ends by terminal deoxynucleotide transferase to visualise the DNA fragments generated by the characteristic endonucleolytic cleavage which occurs during apoptosis. As shown in Figures 1c and 1d, the over expression of Brn-3a resulted in a 3 fold decrease in the number of TUNEL positive cells as compared to the cell line over expressing the vector alone, at 24 hours (45±%, $p<0.005$), 48 hours (35±6%, $p<0.001$) and 72 hours following transfer to serum free media containing RA (37±8%, $p<0.001$). As expected a high proportion of Brn-3a expressing which survived in serum free media containing RA were observed to be process bearing.

Taken together these findings indicate that stable over expression of Brn-3a can protect ND7 cells from an apoptotic fate following one type of differentiation stimulus, and that a proportion of rescued cells are able to differentiate normally. Our previous experiments demonstrate that the ability of Brn-3a to activate its target promoters is dependent on the presence of two activation domains which are absent in Brn-3b. Thus the activation of the tk-Oct test promoter (containing a binding site for the Brn-3 factors upstream of the herpes simplex virus thymidine kinase promoter) requires only the DNA binding POU domain which also acts as an activation domain (Morris *et al.*, 1994). In contrast, this domain has no effect on the α-internexin promoter, which requires a distinct domain at the N-terminus of Brn-3a for promoter activation (Budhram-Mahadeo *et al.*, 1995). Interestingly, both the maximal activation of the SNAP-25 promoter and the ability of Brn-3a to induce process outgrowth to maximal effect requires the presence of both domains, although the POU domain alone is able to confer a less than maximal response (Morris *et al.*, 1997; Smith *et al.*, 1997a).

To test the effects of each of these domains on the ability of Brn-3a to rescue ND7

cells, we prepared pJ5-based stable cell lines overexpressing the isolated POU domain of Brn-3a; or a naturally occuring short form of Brn-3a (Theil *et al.*, 1993) which lacks the N-terminal activation domain (Smith *et al.*, 1997a). When these cell lines were analysed for their ability to rescue ND7 cells from apoptotic PCD, neither the Brn-3a POU domain alone or the short form of Brn-3a were able to confer the protection afforded by full-length Brn-3a (Table I and Figure 2b). Hence the ability of Brn-3a to protect ND7 cells from serum removal induced apoptosis is dependent upon the presence of the N-terminal activation domain that is present in full-length Brn-3a.

We have shown previously that a difference at position 22 in the POU homeodomains of Brn-3a and Brn-3b plays a critical role in the opposite effects of these two isoforms on some target promoters and upon neurite outgrowth. In particular, a construct in which the valine at position 22 of the homeodomain of Brn-3a is replaced with isoleucine (Brn-3aI construct) is no longer able to activate the tk-Oct test promoter in cotransfection assays (Dawson *et al.*, 1996), and is much less able to induce process outgrowth when overexpressed in stable ND7 cell lines (Smith *et al.*, 1997a).

To further extend the observation that the N-terminal domain of Brn-3a is apparently critical for Brn-3a to the rescue ND7 cells from apoptosis, we constructed stable cell lines capable of overexpressing these mutant forms of Brn-3a (Brn-3aI) and Brn-3b (Brn-3bV). The Brn-3aI cell line, in which the expressed protein contains the N-terminus of Brn-3a but the functional POU domain of Brn-3b, maintained the ability to rescue ND7 cells when assayed by all criteria described above (Table I). This contrasts with the inability of this construct to induce neurite outgrowth due to the lack of a functional Brn-3a POU domain (Smith *et al.*, 1997a). Conversely, the overexpression of the Brn-3bV construct which contains a functional Brn-3a POU domain but in conjuction with the N-terminus of Brn-3b (Brn-3bV), was unable to rescue ND7 cells (Table I).

Example 3

A. Brn-3a enhances Bcl-2 protein expression in ND7 cells

To explore the possible mechanisms by which the protection against apoptosis could be conferred by Brn-3a, Western blot analysis was performed on protein extracts from the various cell lines following the observation that Bcl-2 protein level increased approximately four-fold upon differentiation of parental ND7 cells. Incubation with a Bcl-2 specific antibody revealed a sixteen-fold increase in Bcl-2 protein levels in Brn-3a

(long form) over expressing cell lines compared to those expressing vector alone (Figure 3a top panel and Figure 3b). No significant increase in Bcl-2 protein was observed in extracts from cells induced to over express Brn-3b (Figure 3a and 3b). No significant changes in Bcl-2 protein levels were observed in vector control cells indicating that the addition of dexamethasone to the media does not regulate Bcl-2 in ND7 cells (Figure 3a top panel).

Investigation of protein extracts from the cell lines overexpressing the mutagenised forms of Brn-3a (Brn-3al) and Brn-3b (Brn-3bV) revealed that mutation at position 22 did not significantly affect the ability of Brn-3a to induce Bcl-2 protein levels, whilst the converse mutation of Brn-3b did not enable this factor to alter Bcl-2 protein levels . Together these data suggest that the activation domain of Brn-3a responsible for regulation of the Bcl-2 gene is likely to be external to the POU domain.

This hypothesis was subsequently confirmed upon analysis of protein extracts from cell lines overexpressing either the isolated Brn-3a POU domain or the naturally occurring short form of Brn-3a. Stable overexpression of either of these two constructs did not significantly alter Bcl-2 protein levels , thus demonstrating that the regulation of Bcl-2 protein expression by Brn-3a is dependent on the N-terminal activation domain present in Brn-3a (long form) paralleling the requirement for this domain in conferring protection from apoptosis in these cells .

B. Brn-3a does not regulate other members of the Bcl-2 family

No significant alterations in the level of Bad or Bax protein expression was observed in any of the cell lines, whilst neither isoform of Bcl-x (Bcl-x_L or Bcl-x_s) was expressed at detectable levels in these cells .

C. Regulation of the Bcl-2 promoter by Brn-3

The 5' flanking sequence of the human Bcl-2 gene contains two distinct promoters (P1 and P2) that are required for initiation of transcription (Figure 4a). Although it is not known which of these two promoter regions is utilised in cells of neuronal origin, studies have demonstrated that in other cell types in which Bcl-2 promoter activity has been characterised (predominately cells of haemopoetic lineage) transcription is predominately initiated from the P1 region. Bcl-2 transcription in the ND7 neuronal cell line used in this study is predominately initiated from within the P2 promoter region in both the undifferentiated and differentiated state (unpublished data).

To examine the regulation of the Bcl-2 promoter by the Brn-3 family members, plasmid constructs containing either the P1 or P2 promoters driving the luciferase reporter gene (Figure 4a) (Chen and Boxer, 1995) were cotransfected into parental ND7 cells with constitutive expression vectors containing either Brn-3a (long form), Brn-3a (short form) or Brn-3b.

In the parental ND7 cell line, both promoter constructs (P1 and P2) were significantly activated by the long form of Brn-3a (Figures 4b and c; $p<0.01$ in all cases) although the P2 construct was activated by Brn-3a to a much greater degree that observed with the P1 construct. The short form of Brn-3a also activated the P1 reporter construct but only to approximately one fifth of the activity conferred by Brn-3a long (Figure 4b; $p<0.05$). Both Brn-3b and the short form of Brn-3a had no significant effect on the P2 promoter construct, although Brn-3b did significantly repress the basal activity of the P1 promoter (Figure 4b: $p<0.01$).

By contrast in BHK cell cotransfection experiments no significant effect on the activity of the P1 promoter reporter gene construct was observed following cotransfection with Brn-3a or Brn-3b (Figure 4b). A small but significant increase in the P2 (250±21%, $p<0.01$) reporter gene construct was however observed after cotransfection with Brn-3a, but these effects were significantly less than those observed in ND7 experiments (data not shown, $p<0.001$ and $p<0.005$, respectively). No effects were observed following cotransfection of Brn-3b with either promoter construct into BHK cells (Figure 4b and c). Therefore, these data demonstrate that the Bcl-2 promoters are activated much more (approximately 10 fold higher) in ND7 cells compared to BHK cells, suggesting a neuronal specific effect. Taken together with the data from ND7 cotransfections, these data also suggest a much stronger activation potential of Brn-3a for the construct containing the P2 promoter region and the sequences immediately 5' to this region in cells of neuronal origin. Importantly, no significant differences between the basal activities of these reporter constructs in ND7 and BHK cells was observed.

The specificity of these effects to the neuronally expressed Brn-3 family of POU factors was confirmed by co-transfection experiments using the Bcl-2 promoter construct in combination with constructs expressing Oct-1, Oct-2.1, Oct-2.4 and Oct-2.5,. No activation or repression of Bcl-2 promoter activity was observed upon co-transfection of these Type II POU factors into either ND7 or BHK cells .

D. The 5' and 3' Bcl-2 promoters are regulated by the N-terminal activation domain of the long form of Brn-3a

To investigate the difference in the levels of activation of the reporter gene constructs by the long and short forms of Brn-3a, the Bcl-2 promoter constructs were co-transfected with expression plasmids containing the Brn-3a POU domain alone, or chimeric Brn-3 constructs generated by the substitution of regions of Brn-3a with the corresponding regions of Brn-3b (Figure 2a).

The isolated POU domain of Brn-3a was not sufficient to confer regulation on Bcl-2 promoter activity (Figures 4b and c) extending the data obtained from the homeodomain position 22 mutants. Moreover, using previously described chimeric constructs containing various combinations of domains of Brn-3a and Brn-3b (Figure 2a) we wished to further characterise the regions of Brn-3a necessary for the activation of the Bcl-2 promoter. Replacement of either the POU domain region (chimera 1), the region immediately 5' to the POU domain (chimera 2), or both these regions (chimera 3) of Brn-3a with the corresponding region of Brn-3b but retaining the N-terminal domain of Brn-3a did not significantly alter the ability of these chimeric constructs to activate the reporter constructs in the manner of Brn-3a (long form) (Figures 4b and c). In contrast, a chimera containing the POU domain of Brn-3a but with the N-terminal region of Brn-3b (chimera 4) was not capable of activation (Figures 4b and c). Taken together these data extend the previous observations and demonstrate that the N-terminal activation domain, and not the POU activation domain, is necessary for activation of the Bcl-2 promoter, although the POU domain is necessary for DNA binding. This result parallels the observation that the N-terminal domain is necessary for activation of endogenous Bcl-2 expression as well as protection of ND7 cells from apoptosis.

E. A potential Brn-3a binding site in Bcl-2 is sufficient for transactivation

To identify the sequence elements within the P1 and P2 regions necessary for transctivation by Brn-3a, deletion constructs of the two Bcl-2 promoters were cloned upstream of the luciferase reporter gene and were cotransfected into parental ND7 cells together with the Brn-3a expression plasmid. Deletion of the P1 promoter revealed a 113bp fragment (-1412 to -1525) necessary for transactivation by Brn-3a (Figure 5a), and which contained a repeat element CCTCTTACTTC (SEQ ID. No. 5) similar to the CTTCNNNCTTC (SEQ ID. No. 6) motif within the internexin promoter that has previously

been shown to bind Brn-3a and Brn-3b (Budhram-Mahadeo *et al.*, 1995), and which is only activated via an N-terminal activation domain present in the long form of Brn-3a.

Investigation of the region containing the P2 Bcl-2 promoter revealed a 569 bp region (-746 to -178) immediately 5' to the region containing the intron and NRE (Young and Korsemeyer, 1993), that was necessary for transactivation by Brn-3a (Figure 5a). This region contains two repeat elements, CTTCTGCTTC (SEQ ID. No. 7) and CATCAATCTTC (SEQ ID. No. 8) similar in sequence to the internexin CTTCNNNCTTC (SEQ ID. No. 6) motif identified in α-internexin. A 173 bp *Bgl*II/*Nsi*I fragment containing this region was excised and subcloned into the chloroamphenicol acetyltransferase reporter gene vector pBL2CAT, and subsequently co-transfected into parental ND7 cells with the Brn-3 expression plasmids. Co-transfection with Brn-3a and Brn-3aI resulted in significant elevation of CAT reporter gene activity (both $p<0.05$), whilst Brn-3b and Brn-3bV did not result in any signlficant alteration in reporter gene activity (Figure 6a).

F. Brn-3a binds to elements within the P1 and P2 Bcl-2 promoters

To confirm that the Brn-3 factors were capable of binding these sequence elements, the synthetic oligonucleotides containing the sequence CCTCTTACTTC (SEQ ID. No. 5) from the P1 promoter (OligoP1) and the sequence CATCAATCTTC (SEQ ID. No. 8) from the P2 promoter (OligoP2) were used for electromobility shift assay.

Both double stranded oligonucleotides (OligoP1 and OligoP2) bound to *in vitro* translated Brn-3a although the affinity of Brn-3a for OligoP2 was approximately 10-fold stronger than for P1 as determined by competition with unlabelled oligo . Specificity of binding was confirmed by competition for binding sites in presence of an excess of unlabelled oligonucleotide. Using individual oligonucleotides as single stranded probes revealed that in both cases Brn-3a bound to the antisense sequence with much greater affinity that to the sense strand . Such an observation is consistent with our previous studies of the α-internexin promoter where we reported that Brn-3a similarly bound the antisense strand containing the CTTCNNNCTTC (SEQ ID. No. 6) motif with greater affinity that it bound the corresponding sense strand (Budhram-Mahadeo *et al.*, 1995).

To determine if these elements alone were sufficient to confer Brn-3 mediated responsiveness, both oligonucleotides were cloned into the pBL2CAT reporter gene vector. The elements contained in these oligonucleotides were thus 5'-CCTCTTACTTC-3' (-1493 to -1503) of P1 (OligoP1) (SEQ ID. No. 5), and 5'-CATCAATCTTC-3' (-584 to -594) of

the region containing P2 (OligoP2) (SEQ ID. No. 8). Analysis of extracts from cells co-transfected with these constructs together with Brn-3 expression vectors demonstrated that the element contained within OligoP2 was sufficient to confer responsiveness to Brn-3a (Figure 6b) although the degree of activation by Brn-3a of this construct was considerably less than previously observed in the experiments using either the full length promoter (compare with Figure 4c) or the isolated 173bp BglII/NsiI fragment (compare with Figure 6a). The isolated element contained within OligoP1 was not sufficient to confer Brn-3a responsiveness .

G. The bax gene promoter is not regulated by Brn-3

To determine whether the transctivation of the Bcl-2 promoter by Brn-3a was a feature of other Bcl-2 related genes, a plasmid containing -687 to -318bp of the human bax promoter region cloned upstream of the CAT reporter gene (clone TM-668; Miyashita and Reed, 1995) was co-transfected into ND7 and BHK cells together with the various Brn-3 expression plasmids. No significant elevation or repression of CAT reporter gene expression levels was observed in all experiments .

Example 4 – The long form of Brn-3a rescues primary DRG and cerebellar neurons from apoptosis

To extend the *in vivo* relevance of these findings Brn-3 expression constructs were microinjected into primary cultured neonatal DRG neurons together with a lacZ expressing marker plasmid, and protection from NGF withdrawal-induced apoptosis assessed, and effects on cultured cerebellar neurons following glutamate induced apoptosis measured. These experiments showed a similar and highly statistically significant reduction in the numbers of cells undergoing apoptosis following Brn-3a expression which was not shown after expression of Brn-3b (see below).

Materials and Methods

DRGs were dissected from newborn Wistar rat pups at postnatal day 1 (P1). Following the removal of connective tissue sheaths, cells were dissociated in collagenase (0.3%) and subsequently seeded onto poly-ornithine/laminin coated glass coverslips at a final density of 200-250 neurons per coverslip. Cells were cultured in defined medium (Davies, 1995) supplemented with recombinant NGF at a final concentration of 20 ng/ml.

The following day, the number of neurons present on each coverslip was determined by standard microscopic means. Neurons were microinjected with 100 ng/cell of pCi (Promega) expression vector containing murine long form Brn-3a cloned downstream of the constitutive cytomegalovirus gene promoter. Control neurons were injected with 100 ng/cell of empty pCi expression vector. Microinjections were made into the nuclei of cells, and all neurons on the coverslips were injected.

Cultures were incubated for a further 24 hours before media was replaced with fresh media that did not contain NGF. In order to determine the amount of cell death in cultures containing NGF, the media in duplicate coverslips was replaced with media supplemented with NGF. The number of neurons on each coverslip was determined 24 hours following NGF withdrawal.

Cell survival was calculated for each sample as the number of neurons post-injection as a proportion of the pre-injection number.

Results

To investigate the potential role for Brn-3a in the regulation of the survival of developing neurons we analysed the effect of over expression of this factor in primary cultures of NGF-dependent postnatal DRG neurons. Over expression of Brn-3a enhanced the survival of DRG neurons deprived of NGF (Figure 7a, Table II). Thus the long form of Brn-3a can promote the survival of NGF deprived DRG neurons and that this effect is dominant to that of NGF.

Table II. Brn-3a overexpression protects neonatal dorsal root ganglion neurons from apoptosis induced by withdrawal of nerve growth factor.

Injected Construct	Total Number of Cells	
	+NGF	-NGF
Vector	220	126
Brn3a	236	220

Similar results were obtained in primary cultures of sensory neurons obtained from neonatal rat trigeminal ganglia cultured and treated in an identical manner (Fig 7b, Table III). This population of neurons are also dependent on NGF for survival *in vitro*.

Table III. Brn-3a over expression protects neonatal trigeminal ganglion neurons from apoptosis induced by withdrawal of nerve growth factor.

Injected Construct	Total Number of Cells	
	+NGF	-NGF
Vector	125	40
Brn3a	125	95

Example 5 – Expression of Brn-3a in DRG neurons using a disabled herpes simplex viral vector.

Materials and Methods

Culture of Primary Neurons

Dorsal root, trigeminal and superior cervical ganglia were dissected from newborn Sprague Dawley rat pups at postnatal day 1 (P1) or staged C57 mouse embryos where the day of finding the vaginal plug was designated embryonic day e0.5. Following the removal of connective tissue sheaths, ganglia were incubated in trypsin (0.1% (Worthington) in calcium and magnesium-free Earles Balanced Salt Solution). Ganglia were then washed twice in PBS, dissociated and plated onto sterile glass coverslips precoated with polyornithine (0.5 mg/ml) and laminin (20 µg/ml) at a final density of approximately 200 neurons per coverslip. Cells were cultured in defined medium supplemented with recombinant nerve growth factor (NGF; Gibco) at a final concentration of 20 ng/ml.

The following day, 1µg of pCi expression vector containing the murine long form of Brn-3a cloned downstream of the constitutive cytomegalovirus (CMV) gene promoter (pCi3A) was introduced into cultured cells by liposome-mediated transfection. Control cultures were similarly transfected with 1 µg of empty pCi expression vector (pCi). Transfected cells were incubated for 24 hours following transfection and media was then replaced with fresh medium with or without the addition of NGF. To determine efficiency of transfection and to identify individual transfected neurons, cultures were co-transfected with 1 µg of plasmid encoding the lacZ gene under the control of the CMV promoter (CMV-LacZ). Survival of transfected cells (as visualized by β-galactosidase staining) was assessed twenty four hours after the transfer to medium with or without NGF. In parallel

experiments 100 ng of plasmid DNA (pCi or pCi3A together with CMV-LacZ) was introduced into nuclei of neurons by microinjection.

Virus Construction and Growth

Plasmid pR20.5 consists of an RSV/LacZ/pA and a CMV/GFP/pA cassette in opposite (back-to-back) orientations separated by a central region derived from the HSV genome (nts 118,866-120,219) which allows expression of both the RVS and CMV driven genes during HSV latency. These sequences can be excised from the pGEM5 (Promega) plasmid backbone with SrfI since an oligonucleotide containing a SrfI site was inserted on either side of this cassette. The RSV promoter was excised from pRc/RSV (Invitrogen), lacZ/pA from pCH110 (Pharmacia), CMV/pA from pcDNA3 (Invitrogen) and GFP from pEGFP-N1 (Clontech) for the construction of plasmid pR20.5.

The RSV/LacZ/pA and CMV/GFP/pA expression cassette was excised from pR20.5 by digestion with SrfI and sub-cloned into the unique NsiI site of a shuttle vector containing herpes simplex virus 1 (HSV-1) UL43 gene sequences (Coffin *et al.*, 1996) so that the cassette was flanked by UL43 gene sequences. This gave plasmid pR20.5/UL43. The full length Brn-3a cDNA was cloned into the BamH1 site of plasmid pR20.5/UL43, downstream of the Rous sarcoma virus (RSV) promoter, and in reverse orientation to the cDNA encoding green fluorescent protein (GFP) cloned under the control of the CMV promoter. The resulting pR20.5/Brn-3a/UL43 shuttle vector was then co-transfected into BHK cells together with HSV-1 strain 1764 DNA which contains an inactivating insertion in the Vmw65 gene as well as deletion of both copies of the ICP34.5 gene (Coffin *et al.*, 1996b). Recombinant virus was subsequently plaque purified on the basis of the visualisation of GFP under ultraviolet conditions. Western blot analysis was performed to confirm that high levels of Brn-3a protein were produced in infected cultures before a high titre stock was grown. Control virus containing the bacterial lacZ gene under the RSV promoter (that is, containing pR20.5/lacZ/UL43) was similarly generated but without the Brn-3a cloning step.

Trigeminal ganglion neurons were isolated and maintained in cultures as described above on glass coverslips at a density of approximately 200 neurons per coverslip. Cultures were infected in duplicate with approximately $1x10^5$ plaque forming units (pfu)/coverslip of Brn-3a or control virus for 60 mins, washed and then maintained in medium supplemented with NGF for 24 hours. Efficiency of viral infection was determined by visualisation of

GFP before media was replaced with fresh media with or without the addition of NGF. Neuronal cell counts were then performed 24 hours later.

Statistics

Comparisons between medians were performed with the Mann-Whitney test using Minitab for Windows software.

Results

To demonstrate that viral vectors directing the over-expression of Brn-3a could be introduced efficiently into neuronal cells we prepared a disabled herpes simplex virus (HSV) vector expressing both Brn-3a and the green fluorescent reporter protein (GFP). This vector lacks functional viral genes encoding the Vmw65 transactivator protein and the ICP34.5 neurovirulence factor and we have previously shown that it is able to effectively deliver genes to neuronal cells both *in vitro* and *in vivo* without causing significant neuronal cell death.

Cultures of cervical or lumbar DRGs from E16 embryonic animals were infected with this virus or with a control virus expressing only a reporter gene. The effect on survival in the presence or absence of NGF was assessed as before. In these experiments (Table IV) both the cervical and lumbar DRG cultures showed dramatic neuronal losses upon NGF withdrawal. In both cases, the Brn-3a expressing virus was able to produce significant protection against such loss. These data thus demonstrate that the protective effect of Brn-3a can be observed with a virus vector as well as by liposome-mediated delivery of a Brn-3a expression plasmid.

Table IV Survival of cultures of E16 lumbar or cervical dorsal root ganglion neurons infected with a disabled herpes simplex virus vector expressing green fluorescent protein (vector) or the same virus expressing Brn-3a and then cultured in the presence or absence of NGF. Values are the mean of at least three independent experiments whose standard deviation is shown.

	Lumbar	Cervical
Vector +NGF	98±0	98.5±0.5
Vector -NGF	25.5±1.5	38.5±0.5
Brn 3a +NGF	89.5±8.5	83±8.5
Brn 3a -NGF	71.5±0.5	88±5

Example 6 – Endogenous Brn-3a protects neuronal cells from apoptotic cell death

Materials and Methods

Plasmid constructs

Full length Brn-3a cDNA expression vectors are described in Smith *et al.*, 1997c, Lakin *et al.*, 1995 and Budhram-Mahadeo *et al.*, 1995. Antisense Brn-3a specific construct was generated by polymerase chain reaction (PCR) using the primers GGATCCGCTGCAG AGCAACCTCTTC (SEQ ID No. 9) and GTCGACGAGCGA CGGCGACGAGATC (SEQ ID No. 10) where the bold and underlined sequences indicate BamHI and SalI restriction enzyme recognition sites respectively. The 240 base pair PCR product was ligated into pGEM-T vector (Promega, UK) and subcloned as a BamHI/SalI fragment into the mammalian expression vector pJ7 (Morgenstern,and Land, 1990b)

Results

We have shown in the previous examples that over-expression of Brn-3a plays a role in the regulation of the survival of developing neurons. To determine whether endogenous Brn-3a expression also plays a role in the survival of these neurons, an anti-sense approach was used. Thus, a specific anti-sense construct was generated in which a 240 base pair region derived from the N-terminus of Brn-3a which cannot cross-hybridize to either Brn-3b or Brn-3c was expressed in an anti-sense orientation under the control of the CMV promoter. We have previously used a similar approach to successfully reduce the level of Brn-3a in an immortalised neuronal cell line (Lakin *et al.*, 1995).

Trigeminal ganglion neurons obtained from rat pups at post natal day one were cultured for 24 hours in medium supplemented with NGF and plasmid DNA was then introduced by liposome-mediated transfection. Parallel samples of cells were transfected

with either (i) empty expression vector lacking any insert, (ii) the same expression vector containing full length cDNA for Brn-3a cloned downstream of the constitutive cytomegalovirus gene promoter or (iii) the same expression vector containing the 240 bp anti-sense construct derived from the N-terminus of Brn-3a. Each transfection also included a β-galactosidase expression vector allowing successfully transfected cells to be visualized, by staining for β-galactosidase. Typical transfection efficiency was observed to be between 60-70%. The cell survival was then calculated for each sample on the basis of the number of surviving transfected neurons in each case.

In these experiments, as before, a clear enhancement of survival in the cells transfected with Brn-3a was observed compared to those transfected with vector even in the presence of NGF (Tables V and VI). However, importantly, both trigeminal (Table V) and DRG (Table VI) neurons showed significantly reduced survival when transfected with the Brn-3a anti-sense construct compared to the transfection with empty expression vector. This reduced survival was observed both in the presence or absence of NGF, indicating that expression of Brn-3a is important for survival under both these conditions ($p<0.05$ when survival of cells transfected with vector is compared to that of cells transfected with the Brn-3a anti-sense plasmid). Interestingly, in this case, the higher survival of DRG neurons in the absence of NGF was paralleled by a greater reduction in their survival when transfected with the Brn-3a anti-sense construct (compare Tables V and VI).

Table V. Survival of cultures of post-natal trigeminal ganglion neurons, transfected with empty expression vector, Brn-3a expression vector or anti-sense Brn-3a vector and then cultured in the presence or absence of NGF. Values are the mean of at least three independent experiments whose standard deviation is shown.

	+NGF	-NGF
Vector	70±3	27±17
Brn 3a	91±5	79±0.5
Brn 3a antisense	35±11	18±9

These data thus indicate that Brn-3a over expression can enhance the survival of both trigeminal and DRG neurons both in the presence and absence of a specific neurotrophic factor.

Table VI Survival of cultures of post-natal dorsal root ganglion neurons, transfected with empty expression vector, Brn-3a expression vector or anti-sense Brn-3a vector and then cultured in the presence or absence of NGF. Values are the mean of at least three independent experiments whose standard deviation is shown.

	+NGF	-NGF
Vector	74.5±11.5	47±5
Brn 3a	84±6	64±17
Brn 3a antisense	37±7	15±3

Example 7 – Over-expression of Brn-3a increases neuronal cell survival at different stages of development.

To extend the studies on the ability of Brn-3a to protect neurons from different stages of development, we carried out similar studies examining the effect of over-expressing Brn-3a on trigeminal ganglion cultures prepared from different embryonic stages from E11 to E18. In these experiments, Brn-3a over-expression had little effect on survival in the presence of NGF compared to the level of survival observed in cells treated with vector alone at the different stages of development examined, presumably because of the high levels of survival which were observed under these conditions (Figure 8a). By contrast, in the absence of NGF, the cultures over expressing Brn-3a showed clearly enhanced survival compared to the cultures treated with vector which was observed at all time points ($p<0.05$ for comparison of Brn-3a or vector treated cells at all time points tested). This indicates that over-expression of Brn-3a can protect trigeminal ganglion neurons at a number of different stages of embryonic development (Figure 8b).

As Brn-3a is expressed in trigeminal ganglia throughout this period of development, we also wish to determine whether anti-sense inhibition of Brn-3a expression would reduce the survival of trigeminal ganglion neurons from different developmental stages. As indicated in Figure 9, anti-sense inhibition of Brn-3a expression led to reduced survival of the trigeminal ganglion neurons in the presence of

NGF when these neurons were derived from the later stages of embryonic development with highly significant differences in survival being observed between the vector control and the Brn-3a anti-sense treated neurons in cultures prepared from day E14 onwards. In contrast, much smaller effects were observed at day E12 and E13 with no effect at all at E11.

Hence, the endogenous Brn-3a which is expressed in neurons at all these stages appears to play a more significant survival-promoting role as development proceeds. This is in agreement with the finding that the trigeminal ganglia of Brn-3a knock out mice appear to be of normal size early in embryonic development and exhibit significant differences in size from those of wild type animals only later during embryonic development and post-natally.

Discussion

We have used the ND7 neuronal cell line as a model system to identify genes whose protein products could protect neuronal cells from apoptosis. In testing genes which might protect neuronal cells from apoptosis we have examined the Brn-3a and Brn-3b transcription factors which were originally identified in our laboratory. The ND7 cell lines artifically over-expressing Brn-3b or the short form of Brn-3a showed no difference from control ND7 cells in the extent of apoptosis following transfer to serum free medium with added retinoic acid indicating that these factors had no protective effect. In contrast the cell lines over-expressing the long form of Brn-3a showed dramatically reduced cell death following such transfer as assayed both by measures of total cell death (the ability to exclude the dye trypan blue) and measures of the degree of apoptosis (the number of cells with less than diploid DNA content, chromatin fragmentation assays and TUNEL labelling to detect free ends of partially degraded DNA).

Hence the long form of Brn-3a but not Brn-3b or the short form of Brn-3a can protect ND7 cells against apoptosis. This effect appears to be associated with the ability of the long form of Brn-3a to induce the expression of the Bcl-2 protein. Thus the ND7 cells expressing the long form of Brn-3a over express Bcl-2 approximately fifteen fold compared to control cells or those expressing the short form of Brn-3a or Brn-3b.

Moreover the *bcl-2* gene promoter is activated by the long form of Brn-3a in co-transfection assays. As is the case for the anti-apoptotic effect, such activation is dependent upon the N-terminal domain of the long form since it is not observed with the

short form of Brn-3a which lacks this domain. In contrast, both the long and short forms of Brn-3a can activate the promoters of genes such as those encoding the neurofilaments and SNAP-25 which are involved in neurite outgrowth and this activation is dependent upon the C-terminal POU domain of the protein which is common to both forms of the proteins. This parallels our finding that the C-terminal domain is essential for inducing neurite outgrowth in ND7 cells and that this effect can thus be induced by both the long and short forms of Brn-3a.

Thus the effects of Brn-3a on protection from apoptosis and stimulation of neurite outgrowth require distinct regions of the protein with the N-terminal domain being required for protection from cell death whilst the C-terminal POU domain stimulates neurite outgrowth. Interestingly, a single mutation in the POU domain of Brn-3a converting a valine residue to the isoleucine found at the equivalent position in Brn-3b abolishes its ability to stimulate genes involved in neurite outgrowth and prevents it from stimulating such outgrowth. However, as expected, this mutation has no effect on the ability of Brn-3a to activate Bcl-2 expression or protect cells from programmed cell death since it does not affect the N-terminal region of the protein. It is therefore possible to prepare forms of Brn-3a with all possible combinations of effects on neurite outgrowth and cell death. The naturally occurring long form of Brn-3a stimulates both neurite outgrowth and survival whilst the short form stimulates only neurite outgrowth. Similarly the long form containing the valine to isoleucine mutation will stimulate survival but not neurite outgrowth whilst the short form containing this mutation has no effect on either process.

These forms of Brn-3a thus offer a unique panel of reagents able to stimulate neurite outgrowth and/or neuronal cell survival acting by stimulating the expression of multiple genes involved in each of these processes. Brn-3a may represent a master regulator inducing specific programmes of gene expression required for neuronal cell survival and process outgrowth. The over expression of Brn-3a is therefore likely to be of more value in a therapeutic situation than the over expression of a single gene encoding a single structural protein involved in these events. Moreover, the availability of forms of Brn-3a which stimulate cell survival and process outgrowth either separately or in parallel would be of value in different situations where it is necessary to stimulate either both these processes or one without the other.

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Theil, T., Zechner, U., Klett, C., Adolph, S. and Moroy, T. (1994). Chromosomal localization and cDNA sequences of the murine Brn-3 family of developmental control genes. *Cytogenetics and Cell Genetics,* 66:267-271

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CLAIMS

1. A polypeptide comprising transcription factor Brn-3a or a derivative thereof for use in a method of treatment of the human or animal body by therapy.

2. A polypeptide according to claim 1 wherein said derivative comprises an N-terminal fragment of Brn-3a.

3. A polypeptide according to claim 1 or 2 wherein said derivative possesses anti-apoptotic activity but not neurite outgrowth stimulating activity.

4. A polynucleotide encoding a polypeptide according to any one of the preceding claims thereof for use in a method of treatment of the human or animal body by therapy.

5. A nucleic acid vector comprising a polynucleotide according to claim 4 operably linked to regulatory sequences permitting expression of said polypeptide in a host cell, for use in a method of treatment of the human or animal body by therapy.

6. A vector according to claim 5 wherein said host cell is a cell of the central or peripheral nervous system of a mammal.

7. A vector according to claim 5 or 6 further comprising mammalian genomic sequences flanking said polynucleotide and regulatory sequences.

8. A vector according to claim 6 or 7 further comprising viral genomic sequences flanking said polynucleotide and regulatory sequences.

9. A viral strain comprising a polynucleotide according to claim 4 operably linked to regulatory sequences permitting expression of a polypeptide encoded by said polynucleotide in a host cell, for use in a method of treatment of the human or animal body by therapy.

10. A viral strain according to claim 9 wherein said viral strain is a herpes simplex virus strain.

11. A composition comprising a polynucleotide according to claim 4, a vector according to any one of claims 5 to 8 or a viral strain according to claim 9 or 10 together with a transfection agent, for use in a method of treatment of the human or animal body by therapy.

12. A composition according to claim 11 wherein said transfection agent is a cationic agent.

13. A composition according to claim 11 wherein said transfection agent is a liposome composition.

14. A composition comprising a polypeptide according to any one of the claims 1 to 3 and at least one other therapeutic polypeptide, for use in a method of treatment of the human or animal body by therapy.

15. A composition comprising a polynucleotide according to claim 3 and at least one other polynucleotide encoding a therapeutic polypeptide, for use in a method of treatment of the human or animal body by therapy.

16. A composition comprising a vector according to any one of claims 5 to 8 and a vector comprising a polynucleotide encoding a second therapeutic polypeptide operably linked to a regulatory sequence permitting expression of said factor in a host cell, for use in a method of treatment of the human or animal body by therapy.

17. A composition comprising a viral strain according to claim 9 or 10 and a viral strain comprising a polynucleotide encoding a second therapeutic polypeptide, for use in a method of treatment of the human or animal body by therapy.

18. A viral strain comprising a polynucleotide encoding a polypeptide according to any one of claims 1 to 3 and a polynucleotide encoding a second therapeutic polypeptide.

19. A composition according to any one of claims 14 to 17 or a viral strain according to claim 18 wherein said second therapeutic polypeptide is a neurotrophic factor.

20. A pharmaceutical composition comprising a polypeptide according to any one of the claims 1 to 3, a polynucleotide according to claim 4, a vector according to any one of claims 5 to 8, a viral strain according to claim 9, 10 or 18, or a composition according to any one of claims 11 to 17 or 19, together with a pharmaceutically acceptable carrier or diluent.

21. A polypeptide according to any one of the claims 1 to 3, a polynucleotide according to claim 4, a vector according to any one of claims 5 to 8, a viral strain according to claim 9, 10 or 18, a composition according to any one of claims 11 to 17 or 19 or a pharmaceutical composition according to claim 20, for use in a method of treatment of a disease of the peripheral or central nervous system of a human or animal.

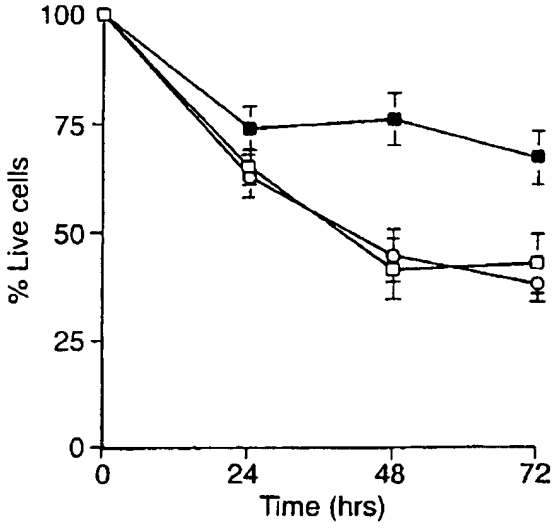
22. A polypeptide, a polynucleotide, a vector, a viral strain or a composition according to claim 21 wherein said disease is a neurogenerative disorder.

23. A polypeptide, a polynucleotide, a vector, a viral strain or a composition according to claim 21wherein said disease is an injury to the peripheral or central nervous system or a human or animal.

24. A polypeptide according to any one of the claims 1 to 3, a polynucleotide according to claim 4, a vector according to any one of claims 5 to 8, a viral strain according to claim 9, 10 or 18, a composition according to any one of claims 11 to 17 or 19 or a pharmaceutical composition according to claim 20, for use in preventing apoptosis in a cell of the peripheral or central nervous system of a human or animal.

25. A polypeptide according to any one of the claims 1 to 3, a polynucleotide according to claim 4, a vector according to any one of claims 5 to 8, a viral strain according to claim 9, 10 or 18, a composition according to any one of claims 11 to 17 or 19 or a pharmaceutical composition according to claim 20, for use in enhancing nerve regeneration following damage.

Fig.1A.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75
50
25
0
% Live cells
0
24
48
72
Time (hrs)

Fig.1B.

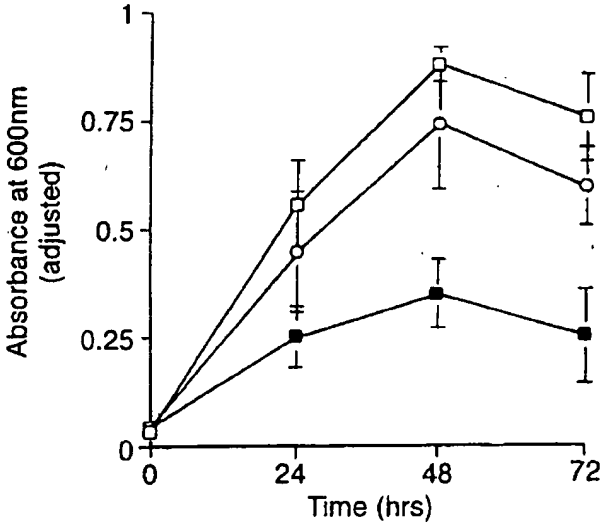

1
0.75
0.5
0.25
0
Absorbance at 600nm (adjusted)
0
24
48
72
Time (hrs)

Fig.1C.

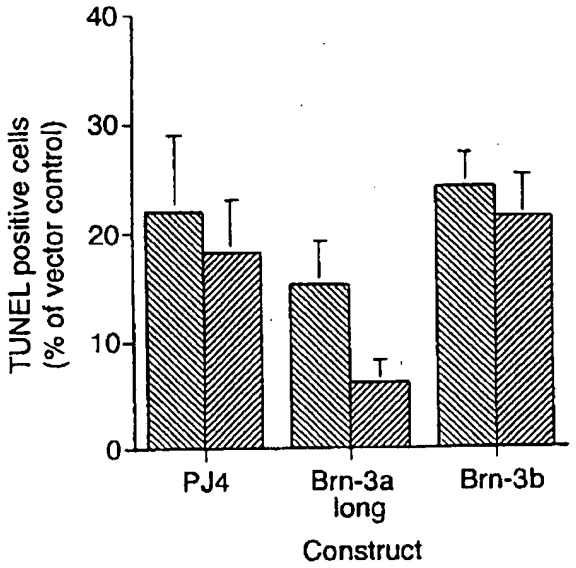

40
30
20
10
0
TUNEL positive cells
(% of vector control)
PJ4
Brn-3a
long
Brn-3b
Construct

Fig.2A.

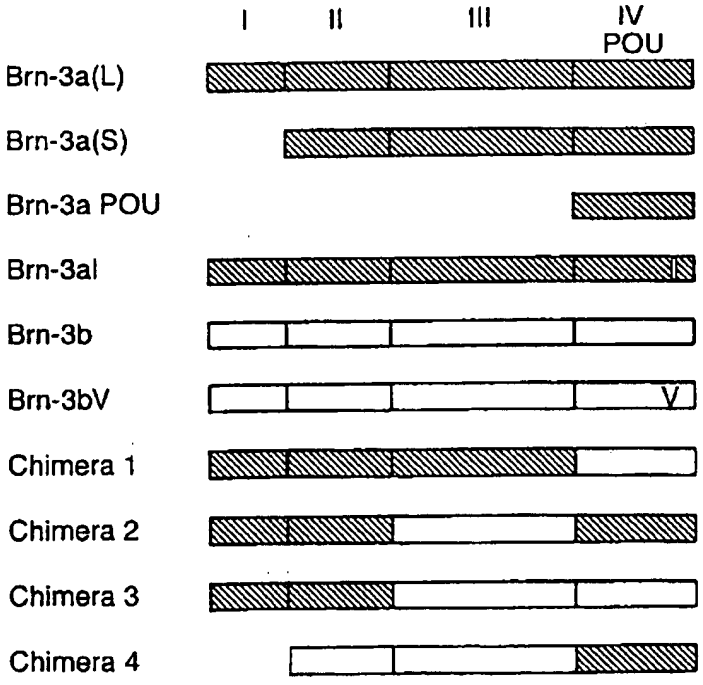

I
II
III
IV
POU
Brn-3a(L)
Brn-3a(S)
Brn-3a POU
Brn-3al
Brn-3b
Brn-3bV
V
Chimera 1
Chimera 2
Chimera 3
Chimera 4

Fig.2B.

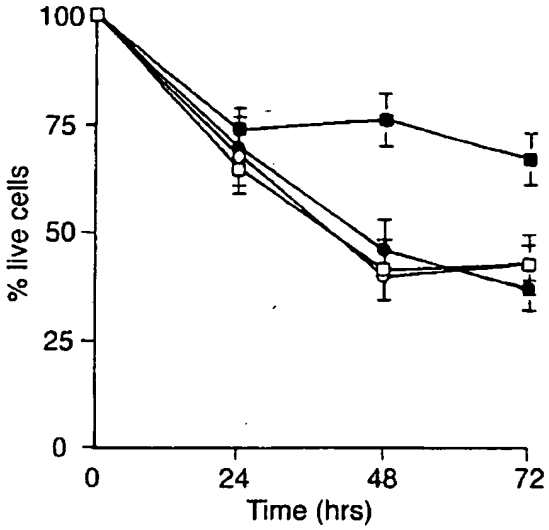

100
75
50
25
0
% live cells
0
24
48
72
Time (hrs)

Fig.3A.

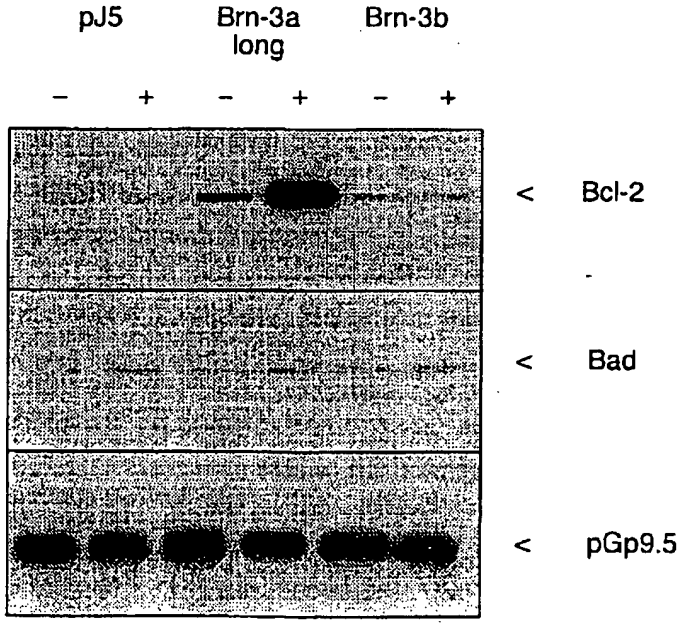

pJ5
Brn-3a long
Brn-3b
– + – + – +
< Bcl-2
< Bad
< pGp9.5

Fig.3B.

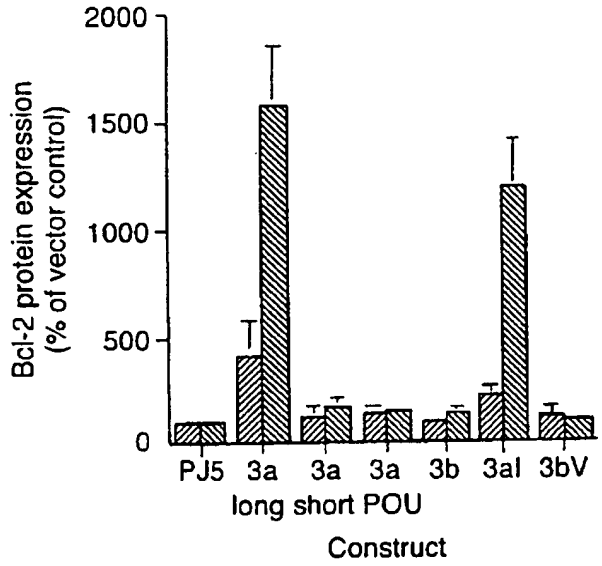


Fig.4A.

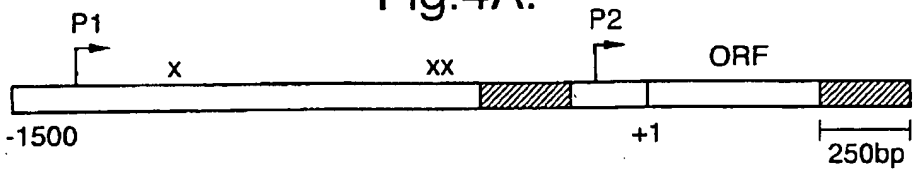


Fig.4B.

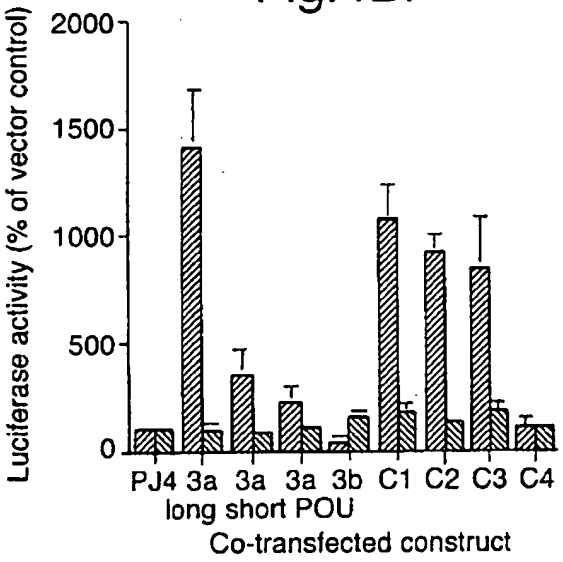


Fig.4C.

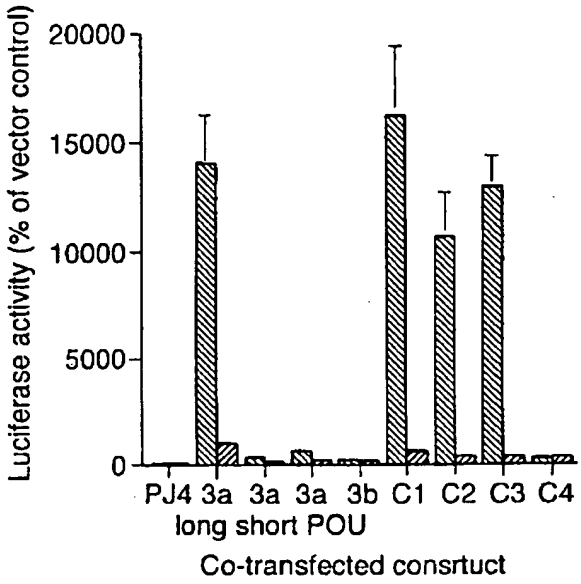
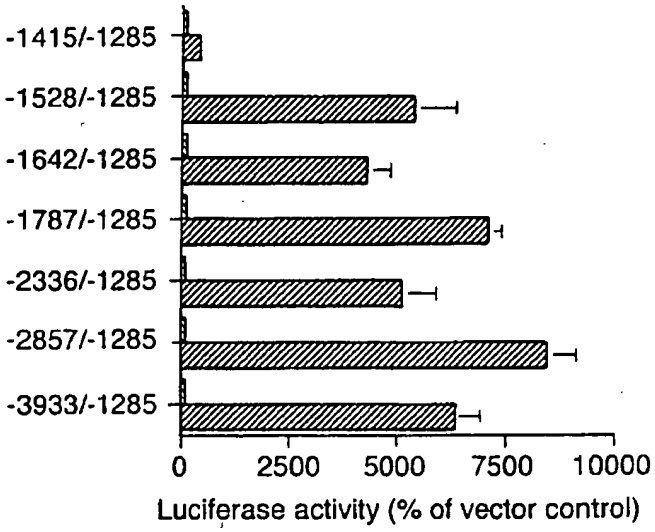

Luciferase activity (% of vector control)
20000
15000
10000
5000
0
PJ4 3a 3a 3a 3b C1 C2 C3 C4
long short POU
Co-transfected consrtuct

Fig.5A.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-1787/-1285
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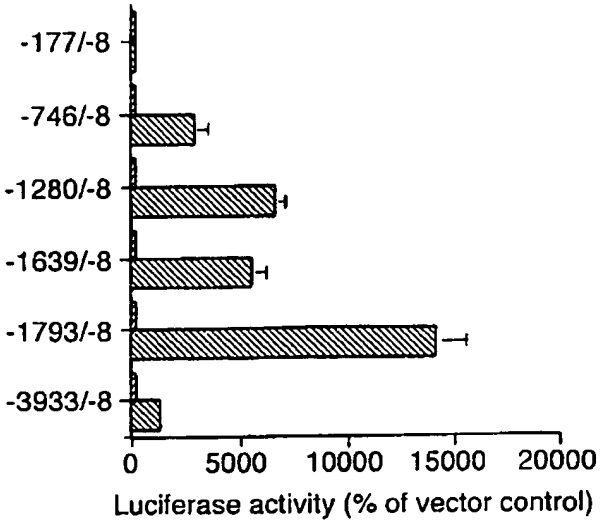
-3933/-1285
0
2500
5000
7500
10000
Luciferase activity (% of vector control)

Fig.5B.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-746/-8
-1280/-8
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0
5000
10000
15000
20000
Luciferase activity (% of vector control)

Fig.6A.

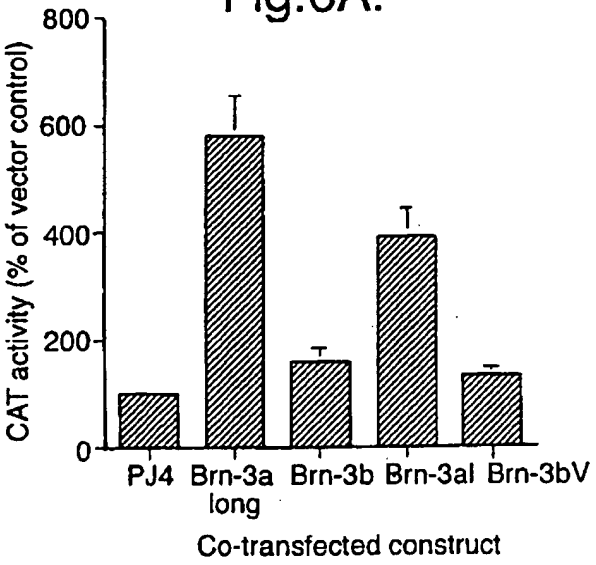

800
600
400
200
0
CAT activity (% of vector control)
PJ4
Brn-3a long
Brn-3b
Brn-3al
Brn-3bV
Co-transfected construct

Fig.6B.

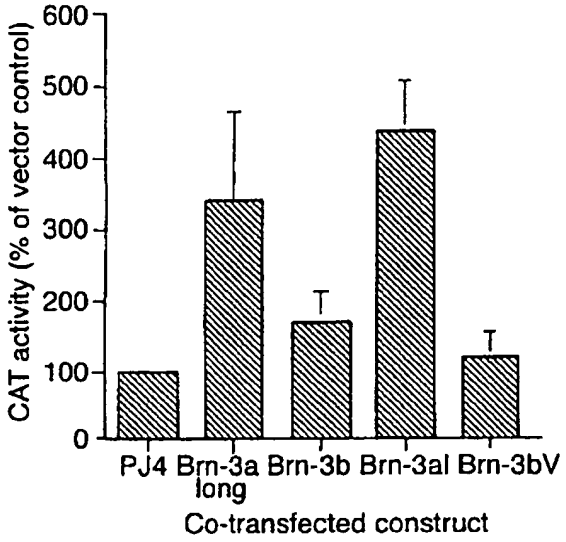
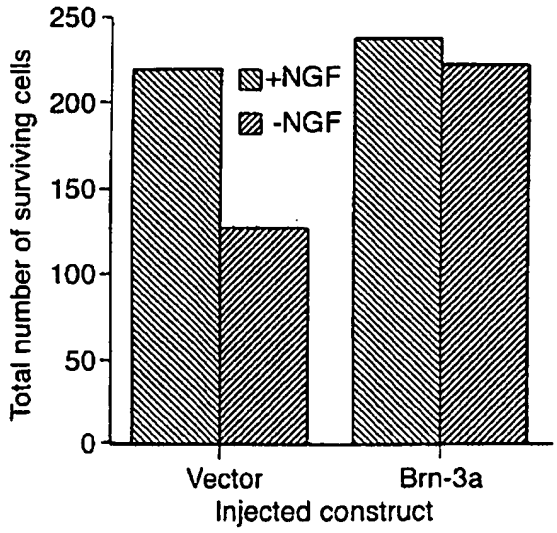

CAT activity (% of vector control)
600
500
400
300
200
100
0
PJ4
Brn-3a long
Brn-3b
Brn-3al
Brn-3bV
Co-transfected construct

Fig.7A.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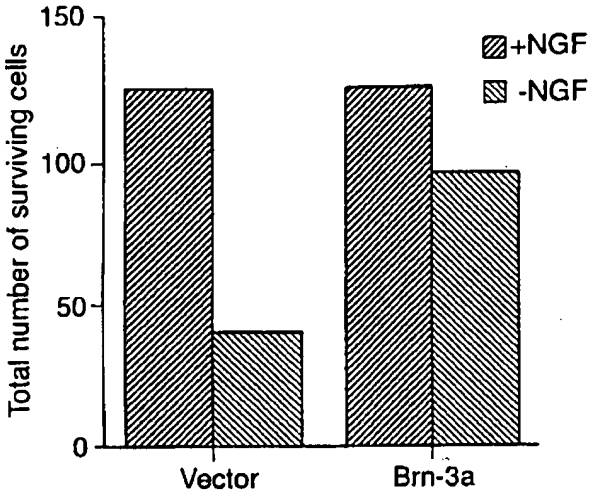
200
150
100
50
0
+NGF
-NGF
Vector
Brn-3a
Injected construct

Fig.7B.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100
50
0
Total number of surviving cells
+NGF
-NGF
Vector
Brn-3a

Fig.8A.

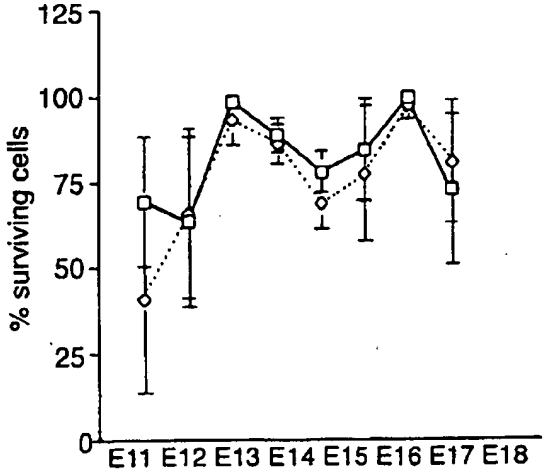

125
100
75
50
25
0
% surviving cells
E11 E12 E13 E14 E15 E16 E17 E18

Fig.8B.

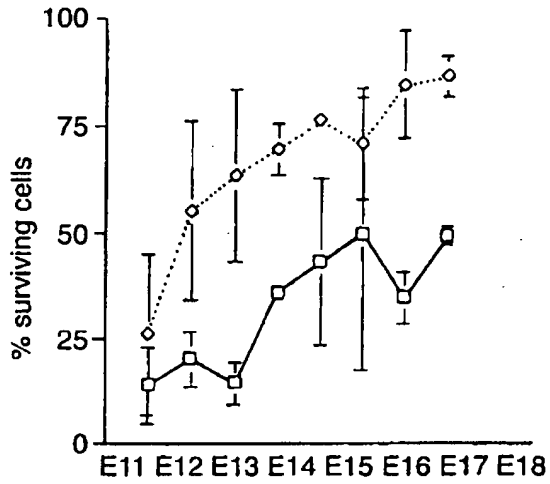
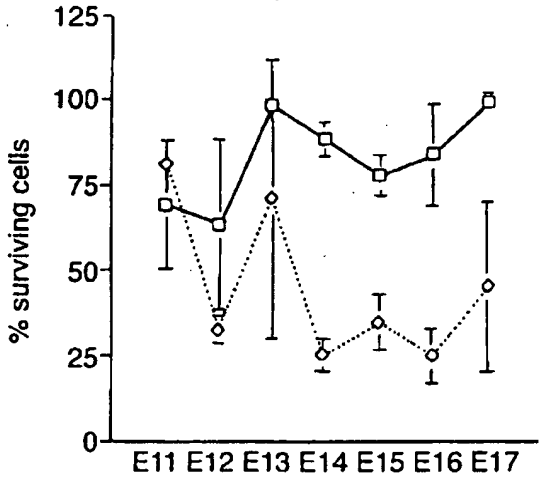

100
75
50
25
0
% surviving cells
E11 E12 E13 E14 E15 E16 E17 E18

Fig.9.


125
100
75
50
25
0
% surviving cells
E11 E12 E13 E14 E15 E16 E17

SEQUENCE LISTING

SEQUENCE DESCRIPTION: SEQ ID NOs: 1 (polynucleotide) and 2 (amino acid):
- human Brn-3a

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            20                  25                  30
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                165                 170                 175
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Pro His Pro His Met His Ser Leu Gly His Leu Ser His Pro Ala Ala
        195                 200                 205
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225                 230                 235                 240
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                245                 250                 255
GCG GGC CTG GCG TCC ATC TGC GAC TCG GAC ACG GAC CCG CGC GAG CTC        816
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            260                 265                 270
GAG GCG TTC GCG GAG CGC TTC AAG CAG CGG CGC ATC AAG CTG GGC GTG        864
Glu Ala Phe Ala Glu Arg Phe Lys Gln Arg Arg Ile Lys Leu Gly Val
        275                 280                 285
ACG CAG GCC GAC GTG GGC TCG GCG CTG GCC AAC CTC AAG ATC CCG GGC        912
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    290                 295                 300
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305                 310                 315                 320
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                325                 330                 335
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            340                 345                 350
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        355                 360                 365
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    370                 375                 380
TCG TCC GAG AAG ATC GCC GCC ATC GCC GAG AAA CTG GAC CTC AAA AAG       1200
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385                 390                 395                 400
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SEQUENCE DESCRIPTION: SEQ ID NOs: 3 (polynucleotide) and 4 (amino acid):
· murine Brn-3a

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Leu Pro Glu His Lys Tyr Pro Ser Leu His Ser Ser Ser Glu Ala Ile
            20                  25                  30
CGG CGG GCC TGC CTG CCC ACG CCG CCG CTG CAG AGC AAC CTC TTC GCC       144
Arg Arg Ala Cys Leu Pro Thr Pro Pro Leu Gln Ser Asn Leu Phe Ala
        35                  40                  45
AGC CTG GAC GAG ACG CTG CTG GCG CGG GCC GAG GCG CTG GCG GCC GTG       192
Ser Leu Asp Glu Thr Leu Leu Ala Arg Ala Glu Ala Leu Ala Ala Val
    50                  55                  60
GAC ATC GCG GTG TCC CAG GGC AAG AGC CAC CCT TTC AAG CCG GAC GCC       240
Asp Ile Ala Val Ser Gln Gly Lys Ser His Pro Phe Lys Pro Asp Ala
65                  70                  75                  80
ACG TAC CAC ACG ATG AAT AGC GTG CCC TGC ACG TCC ACG TCC ACC GTG       288
Thr Tyr His Thr Met Asn Ser Val Pro Cys Thr Ser Thr Ser Thr Val
                85                  90                  95
CCG CTG GCG CAC CAC CAC CAC CAC CAC CAC CAC CAC CAG GCG CTC GAG       336
Pro Leu Ala His His His His His His His His His Gln Ala Leu Glu
            100                 105                 110
CCC GGT GAC CTG CTG GAC CAC ATC TCG TCG CCG TCG CTC GCG CTC ATG       384
Pro Gly Asp Leu Leu Asp His Ile Ser Ser Pro Ser Leu Ala Leu Met
        115                 120                 125
GCC GGC GCA GGG GGC GCA GGC GCG GCG GGA GGC GGC GGC GGC GCC CAC       432
Ala Gly Ala Gly Gly Ala Gly Ala Ala Gly Gly Gly Gly Gly Ala His
    130                 135                 140
GAC GGC CCC GGG GGC GGA GGC GGA CCG GGG GGC GGC GGT GGC CCG GGC       480
Asp Gly Pro Gly Gly Gly Gly Gly Pro Gly Gly Gly Gly Gly Pro Gly
145                 150                 155                 160
GGC GGC GGC CCC GGG GGT GGC GGC GGC GGC GGC GGC CCG GGG GGC GGC       528
Gly Gly Gly Pro Gly Gly Gly Gly Gly Gly Gly Gly Pro Gly Gly Gly
                165                 170                 175
GGC GGC GCC CCG GGC GGC GGG CTC TTG GGC GGC TCG GCG CAT CCG CAC       576
Gly Gly Ala Pro Gly Gly Gly Leu Leu Gly Gly Ser Ala His Pro His
            180                 185                 190
CCG CAC ATG CAC GGC CTG GGC CAC CTG TCG CAC CCC GCG GCG GCG GCG       624
Pro His Met His Gly Leu Gly His Leu Ser His Pro Ala Ala Ala Ala
        195                 200                 205
GCC ATG AAC ATG CCG TCC GGG CTG CCG CAT CCC GGG CTC GTG GCC GCG       672
Ala Met Asn Met Pro Ser Gly Leu Pro His Pro Gly Leu Val Ala Ala
    210                 215                 220
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Ala Ala His His Gly Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Gly
225                 230                 235                 240  CAG GTG
GCG GCG GCG TCG GCC GCG GCG GCG GTG GTG GGC GCG GCG GGC       768
Gln Val Ala Ala Ala Ser Ala Ala Ala Ala Val Val Gly Ala Ala Gly
                245                 250                 255
CTG GCG TCC ATC TGC GAC TCG GAC ACG GAC CCG CGC GAG CTC GAG GCG       816
Leu Ala Ser Ile Cys Asp Ser Asp Thr Asp Pro Arg Glu Leu Glu Ala
            260                 265                 270
TTC GCC GAG CGC TTC AAG CAG CGG CGC ATC AAG CTG GGC GTG ACG CAG       864
Phe Ala Glu Arg Phe Lys Gln Arg Arg Ile Lys Leu Gly Val Thr Gln
        275                 280                 285
GCC GAC GTG GGC TCG GCG CTG GCC AAC CTC AAG ATC CCG GGC GTG GGC       912
Ala Asp Val Gly Ser Ala Leu Ala Asn Leu Lys Ile Pro Gly Val Gly
    290                 295                 300
TCG CTC AGC CAG AGC ACC ATC TGC AGG TTC GAG TCG CTC ACG CTC TCG       960
Ser Leu Ser Gln Ser Thr Ile Cys Arg Phe Glu Ser Leu Thr Leu Ser
305                 310                 315                 320
CAC AAC AAC ATG ATC GCG CTC AAG CCC ATC CTG CAG GCG TGG CTG GAG      1008
His Asn Asn Met Ile Ala Leu Lys Pro Ile Leu Gln Ala Trp Leu Glu
                325                 330                 335
GAG GCC GAG GGC GCG CAG CGT GAG AAA ATG AAC AAG CCG GAG CTC TTC      1056
Glu Ala Glu Gly Ala Gln Arg Glu Lys Met Asn Lys Pro Glu Leu Phe
            340                 345                 350
AAC GGC GGC GAG AAG AAG CGC AAG CGG ACT TCC ATC GCC GCG CCC GAG      1104
Asn Gly Gly Glu Lys Lys Arg Lys Arg Thr Ser Ile Ala Ala Pro Glu
        355                 360                 365
AAG CGC TCC CTC GAG GCC TAT TTT GCC GTA CAA CCC CGG CCC TCG TCT      1152
Lys Arg Ser Leu Glu Ala Tyr Phe Ala Val Gln Pro Arg Pro Ser Ser
    370                 375                 380
GAG AAG ATC GCC GCC ATC GCC GAG AAA CTG GAC CTC AAA AAG AAC GTG      1200
Glu Lys Ile Ala Ala Ile Ala Glu Lys Leu Asp Leu Lys Lys Asn Val
385                 390                 395                 400
GTG CGG GTG TGG TTT TGC AAC CAG AGA CAG AAG CAG AAG CGG ATG AAA      1248
Val Arg Val Trp Phe Cys Asn Gln Arg Gln Lys Gln Lys Arg Met Lys
                405                 410                 415
TTC TCT GCC ACT TAC TGA                                             1266
Phe Ser Ala Thr Tyr
            420
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INTERNATIONAL SEARCH REPORT

Internatic Application No
PCT/GB 98/02228

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/86 C07K14/47 C12N7/00 A61K48/00
A61K39/245 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SMITH M.D. ET AL.: "Inhibition of neuronal process outgrowth and neuronal specific gene activation by the Brn-3b transcription factor" J. BIOL. CHEM., vol. 272, no. 2, 10 January 1997, pages 1382-1388, XP002087056 cited in the application see the whole document	1,2,4-8, 11-13, 21-25
A		3,14-19

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 December 1998	21/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

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INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/GB 98/02228

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BUDHRAM-MAHADEO V. ET AL.: "The different activities of the two activation domains of the Brn-3a transcription factor are dependent on the context of the binding site." J. BIOL. CHEM., vol. 271, no. 15, 12 April 1996, pages 9108-9113, XP002087057 cited in the application see the whole document	1,2,4-8, 11-13, 21-25
X	LAKIN N. ET AL.: "Regulation of neurite outgrowth and SNAP-25 gen expression by the Brn-3a transcription factor" J. BIL. CHEM., vol. 270, no. 26, 30 June 1995, pages 15858-15863, XP002087058 cited in the application see the whole document	1,2,4-8, 11-13, 21-25
X	SMITH, MD ET AL.: "The Brn-3a transcription factor induces neuronal proceses outgrowth and the coordinate expression of genes encoding synaptic proteins." MOL. CELL. BIOL., vol. 17, no. 1, January 1997, pages 345-354, XP002087059 cited in the application see abstract	1,2,4-8, 11-13, 21-25
P,X	SMITH M.D. ET AL.: "Bcl-2 transcription from the proximal P2 promoter is activated in neuronal cells by the Brn-3a POU family transcription factor." J. BIOL. CHEM., vol. 273, no. 27, 3 July 1998, pages 16715-16722, XP002087060 see the whole document	1,2,4-25
P,X	WO 98 04726 A (BROWN SUZANNE MOIRA ;MACLEAN ALASDAIR RODERICK (GB); MEDICAL RES C) 5 February 1998 see page 8 - page 9	9,10

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatio application No
PCT/GB 98/02228

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9804726 A	05-02-1998	AU 3700797 A	20-02-1998

余白頁